FINAL REPORT

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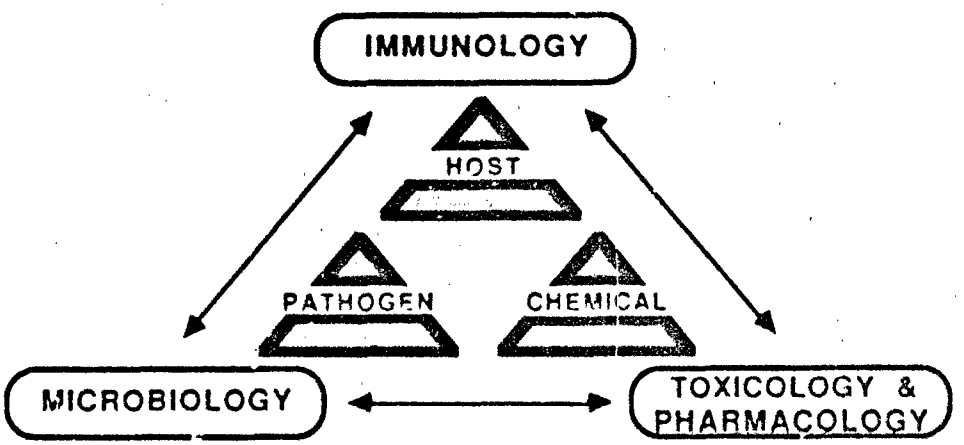
November, 1986

Development of Short Term Immunotoxicological Assays for Prediction of Chronic Toxicological Responses Induced by Environmental Chemicals



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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The overall goal of this research project was to systematically develop a battery of in vitro functional assays to predict subchronic or chronic toxicologic behavior that would be produced by an in vivo exposure to a chemical. The general experimental plan utilized both in vitro and in vivo approaches. The results of the in vivo exposures were used to determine if the in vitro tests could predict the toxicologic behavior. The in vitro studies were developed along a tier system, using spleen and bone marrow cells. The Tier 1 assays for the spleen cells were the direct cytotoxicity as measured by

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trypan blue exclusion and changes in cell number over the exposure period. Als in this tier was the effect of chemicals on DNA synthesis. For Tier 2, the lymphocyte response to a T and a B cell mitogen is used, and in Tier 3, the mor complex cell-cell interactions involved in a Mishell-Dutton assay, the mixed lymphocyte reaction, and the in vitro growth of stem cells are investigated.

The in vivo studies included a complete toxicologic workup in both male and female mice, using trichloroethylene and dexamethasone. Both of these compound were administered in the drinking water. trichloroethylene was administered over a six month period with an interim sacrifice at four months.

For trichloroethylene, the oral LD50 in female mice was 2443 mg/kg (95% confidence limits of 1839-3779 mg/kg) and in male mice was 2402 mg/kg (95% confidence limits of 2065-2771 mg/kg). In the subchronic study, trichloroethylene was administered at concentrations of 0.1, 1.0, 2.5, and 5.0 mg/ml, and mice of both sexes were exposed for four or six months. There was a decreased body weight gain at the highest dose, which could be attributed to a decrease in fluid consumption. The most significant effects attributable to trichloroethylene were an increase in liver weight in both sexes, and an increase in kidney weight in both sexes, accompanied by increases in protein and ketones in the urine. Trichloroethylene failed to elicit any other adverse effects. The only significant effect on immune function was a dose-related decrease in bone marrow stem cells in females.

Based on the more routine toxicological parameters, the most sensitive indicator of dexamethasone toxicity were changes in body, spleen, and thymus weights, depression of peripheral leukocyte counts, and an increase in the percent of polymorphonuclear leukocytes.

Mice exposed to dexamethasone were deficient in humoral and cell-mediated immunity, with minimal effects on the macrophage and bone marrow parameters.

The in vitro studies were able to point to the immune system as a target organ for dexamethasone. However, trichloroethylene effects were not predictable based on the in vitro results.

This report briefly describes the results of the development of the approach and methods now in place for in vitro and in vivo assessment. We believe that this will provide a practical approach to in vitro and in vivo assessment of toxicity, particularly with respect to the immune system.

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EXECUTIVE SUMMARY

Investigations during and subsequent to the experimental period which produced the in vitro approach to toxicological studies were conducted in the Immunotoxicology Program at the Medical College of Virginia/Virginia Commonwealth University. The program coordinator is Albert E. Munson, Ph.D., Professor of Pharmacology and Toxicology. There were three distinct phases to the studies described. The first year of the contract (Phase I) was used to develop a tier concept and to develop an approach to confirm in vitro with in vivo results. The second year (Phase II) was used to select assays and in vitro exposure methods. Subsequent to the second year (Phase III), studies were carried out to refine and choose the best in vitro and in vivo approaches. Phase III studies have provided the present approach to in vitro and in vivo immunotoxicological evaluation. During Phase III, two metabolic activation systems were developed that interface with the in vitro immunological system. One, begun during Phase II, was the 9000 xg liver supernatant, and the second was the primary hepatocyte. Phase II is summarized in this report.

The overall goal for the research performed under this contract was to develop a battery of assays that could be used to predict subchronic and chronic toxicological behavior produced by in vivo exposure to a chemical. Conceptually, in vitro approaches would be more efficient and less expensive than current methods using subchronic and chronic exposures. The plan set forth was to use both in vitro and in vivo approaches, where the results from each would be correlated. Several in vitro assays were investigated and the strengths and weaknesses of each determined. During the initial contract period, several in vitro assays were analyzed and, during the last three years, one in vitro and one in vivo approach have come into focus. Several publications have shown the feasibility of the connection between these two approaches. From the work that was started during the contract period, a process has been formulated to use the in vitro antibody forming cell assays with several variations to provide a first screen for activity. Inherent in the assays is the measurement of the direct cytotoxic action of the xenobiotic.

The cells of the immune system offer the opportunity for the toxicologist/pharmacologist to investigate in vitro the actions of a xenobiotic at several

levels. Cells of the immune system are capable of functioning in vitro as they do in vivo. In a 3 to 7 day period, depending on the antigenic stimulus, immune cells must recognize antigenic determinants (communicated both by physical contact and through humoral mediators - lymphokines) and undergo all the membrane and cytoplasmic transduction events in order to proliferate, produce new messengers, and differentiate into an end line cell that produces a specific product (i.e., antibody or a cell which has the ability to recognize and remove the antigen). With all of these biochemical events ongoing, the addition of the xenobiotic affords it every opportunity to perturb the system. The in vitro approach using the immunocompetent cells also provides the opportunity to determine the cellular site of action of the xenobiotic. Investigations during the contract period focused on several of the specific events, i.e., cell recognition and proliferation. The approach presently taken as a result of all studies in the Immunotoxicology Program is built on a belief that the in vitro antibody forming cell system represents the best assay system.

This report briefly describes the results of the development of the approach and methods now in place for in vitro and in vivo assessment. We believe that this will provide the Department of Defense with a practical approach to in vitro and in vivo assessment of toxicity, particularly with respect to the immune system.

BACKGROUND AND INTRODUCTION

The toxicology community is continuing to face a major problem in fulfilling its responsibility to assess the toxic potential of chemicals before they are registered with federal regulatory agencies. Tests now used to make such assessments are expensive, time-consuming, and not always reliable. The costs of toxicological testing have reduced efforts in chemical and drug discovery.

The high costs of toxicological testing are well-documented. Before a chemical can be registered for human consumption, acute, subchronic, and chronic toxicity studies must be performed in several species of animals at a cost (as of 1976) of $500,000 per compound (Mudel et al., Science. 193:834, 1976). Several governmental agencies conduct toxicological testing at an estimated cost of $100 million and private industry spends much more. Thus, a conservative estimate indicates that approximately 400 compounds can be evaluated per year with the funds derived from federal and private sectors. Data derived from the President's Advisory Committee (Handling of Toxicological Information, Government Printing Office, Washington, DC, 1966) indicate that thousands of chemicals generated from federal and private sources are in need of toxicological evaluation. Based on this information, current resources preclude the evaluation of most chemicals, many of which might be of great benefit or detriment to society.

Even if there were sufficient time and money to evaluate all chemicals being used, there are still complicated issues surrounding the use of in vivo tests: animal, species and strain differences in response to xenobiotics, and meaningful extrapolation of these results to man.

The adverse effects of chemicals on the organs, tissues, and cells of the lymphoreticular system have received considerable attention from both toxicologists and immunologists. This interest is well founded since the immune system has been shown to be the target organ of various chemically and physically diverse compounds. This system, like all others in the body, is complex, with several types of cells working both independently and in concert, to carry out a role in homeostasis. Immunotoxicology is the subject of increased activity and awareness because the physiology and biochemistry of the immune system

are now being systemically dissected; as a result, the basis processes involved in host defense mechanisms are better understood. A second reason for the interest in immunotoxicology is that it represents a system in which the cells from exposed animals can be readily removed and their functions examined in vitro. This is in keeping with the direction in which toxicology is moving, i.e., complementing morphological changes with functional alterations.

The overall aim of this project is to develop a system of in vitro assays that can be used to predict chronic toxicological behavior produced by in vivo exposure to a xenobiotic. Successful development of these in vitro assays will allow for selection of xenobiotics for in vivo toxicological investigation and could provide a basis for the types of in vivo assessment. The in vitro system will allow for the interfacing of biotransforming capability and, possibly, the use of fluids from animals exposed to a xenobiotic. We also recognize that a comprehensive in vitro system requires a strong biochemical basis. A chemical that interferes with high-level cell functions involving specialized, differentiated activities such as antibody synthesis, neurotransmitter production, or insulin synthesis and/or activity, may or may not affect basic functions common to all cells, such as cell growth, enzyme activities or macromolecular synthesis. Therefore, it is important to develop an approach that considers both basic and specialized cell functions. The tier system for in vitro testing that is reported here is based on this approach. Three tiers are outlined according to a sequential level for increasing sensitivity to pertubation by a xenobiotic, i.e., the higher the tier assay, the greater the number of biochemical sites available for alteration. The specific objectives for the first year of the project are listed below:

- Design and develop an in vitro procedure for exposing volatile and non-volatile xenobiotics to bone marrow cells, lymphocytes and macrophages.
- Develop at least two assays that can be defined as cytotoxicity assays.
- Develop two biochemical assays that would signal altered macromolecular metabolism.
- Develop two functional assays that would predict an adverse effect in experimental animals.
- Design, develop and implement subchronic toxicological studies on xenobiotics administered in the drinking water that would provide reference to in vivo investigations.

- Develop analytical capabilities for both the *in vitro* and *in vivo* aspects of the study.

The pathway of investigation for these studies is outlined in Flowchart 1. The chemical is selected for study based on environmental importance, importance to an agency, or its known toxicological effects. The physical and chemical properties provide the basis for designing the exposure vehicle for the *in vitro* and *in vivo* investigations. Acute toxicity data are either generated or obtained from the literature. From the acute toxicity data, a subchronic study is designed and performed. Although studies were performed for up to six months, 14 day subchronic studies may be enough to predict immunotoxicity. The *in vitro* studies involve direct addition of the xenobiotic to bone marrow cells of lymphocytes derived from spleen or thymus, and macrophages derived from the peritoneal cavity. *In vitro* exposure is conducted in the presence and absence of a hepatic metabolizing system. Two xenobiotics (trichloroethylene and dexamethasone) were used in the early development of these assays. A revised tier system which was used is shown below:

I. Tier 1

A) Spleen Cell Number
B) Spleen Cell Viability
   1) ^{51}Chromium release
   2) ^{111}Indium release
   3) Neutral red dye uptake
   4) Trypan blue exclusion
   5) Sensitivity to Pronase
C) Spleen Cell DNA Synthesis

II. Tier 2

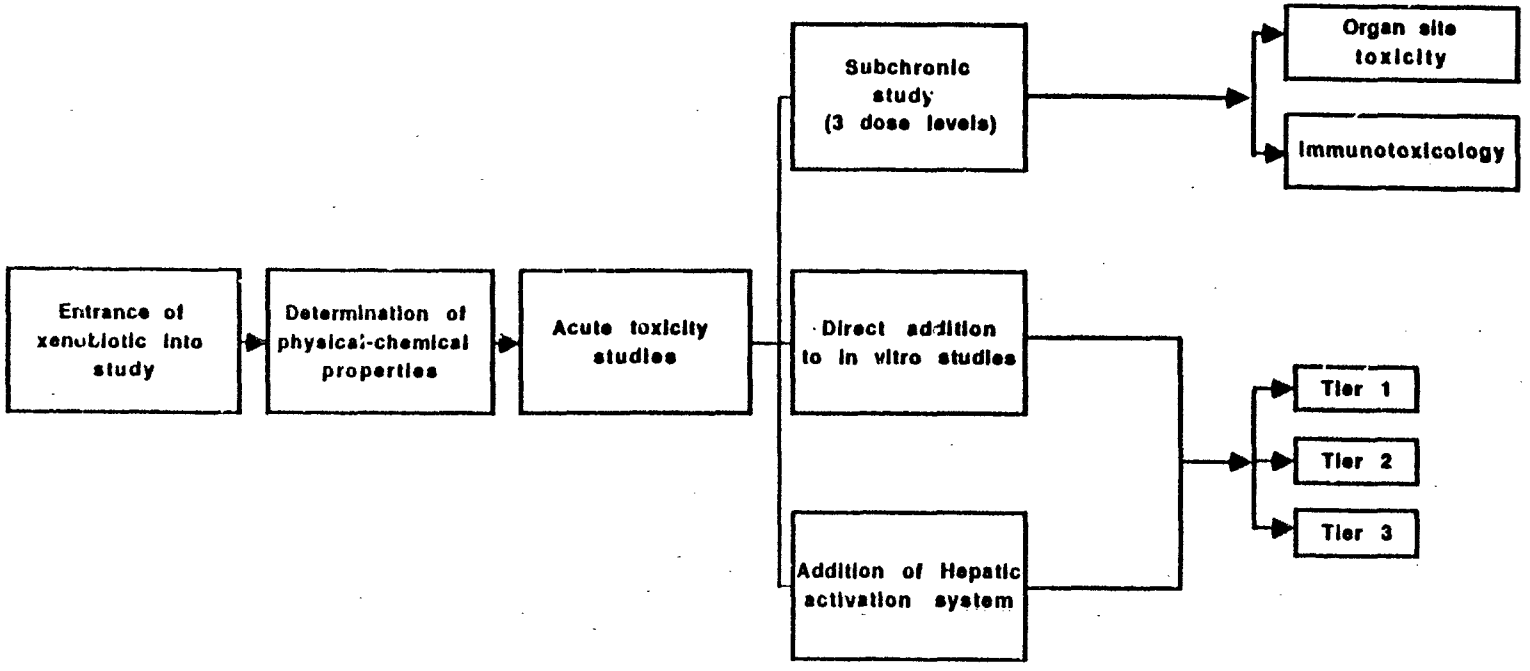
A) Spleen cell response to concanavalin A and bacterial lipopolysaccharide

III. Tier 3

A) IgM antibody response to T-dependent antigen
   1) Mishell-Dutton assay
   2) Mixed lymphocyte reaction
   3) Effects of trichloroethylene
B) Bone marrow parameters
   1) Bone marrow cell number
   2) DNA synthesis
   3) Bone marrow stem cell number (CFU-GM)

Flow Chart 1

PATHWAY OF INVESTIGATION



The Tier one assays are aimed at measuring the cytotoxic effects of the chemicals, the effects on DNA synthesis of resting and stimulated cells and one cellular function, i.e., phagocytosis. Tier two assays involve recognition and functional responses and employ the mixed lymphocyte reaction and macrophage chemotaxis. Tier three assays require information of a product, i.e., stem cell growth and antibody responses. This report describes these assays, most of which were begun during the contract period and have been refined to the present state.

The studies conducted during and subsequent to the contract period have provided a usable and general approach to the development of in vitro assays using immunocompetent cells for assessing perturbations caused by xenobiotics. For Tier 1, Tier 2 and the bone marrow stem cell assay of Tier 3, the cells were exposed to xenobiotics in either 50 ml centrifuge culture tubes or in 250 ml flasks. The larger vessels were used when more cells were required, or when the xenobiotic required greater dilutions because of solubility. Cells were analyzed over a 48 hour period for viability and DNA synthesis in the presence and absence of mitogen. In the other Tier 3 assays, individual exosure were conducted in 96 well culture plates.

As the assays were developed and used, they became somewhat artificial; in the Tier 3 assays, interpretation can be made only if the Tier 1 and Tier 2 assays are included. For example, appropriate analysis of the IgM response requires that cell number, viability and proliferative ability be known. However, the Tier 1 and Tier 2 assays can be performed without Tier 3.

PART I - IN VITRO STUDIES

MATERIALS AND METHODS AND RESULTS

TIER 1

Characterization of the Spleen Cell Assays (Flowchart 2)

Spleen Cell Number

The time course for changes in spleen cell number is shown in Figure 1 and Table 1. Four experiments are shown, with two of the four having viability data. Spleen cells are stable with respect to cell number and viability (pronase method) over the 48 hour experimental period. Cell number did change if the glassware was not siliconized.

Spleen Cell Viability

Several procedures were investigated to determine the best method for assessing cell viability. Five methods were studied: ^{51}chromium release, ^{111}indium release, neutral red dye uptake, trypan blue exclusion and susceptibility to pronase.

^{51}Chromium Release: Bone marrow, and spleen and thymus cells were radiolabeled with sodium ^{51}chromium. The radioactive label enters the cell's cytoplasm and is released upon lysis of the cell. This is a frequently used assay for measuring cytotoxicity induced by natural killer cells and for T cell mediated cytotoxicity. For the most part, these assays are completed in 4 hours. For *in vitro* toxicity studies, 48 hours was set as the time for exposure to the xenobiotic. Spontaneous release was highly variable from experiment to experiment and sensitivity was not as good as with trypan blue or the pronase method. Five experiments were conducted using ^{51}chromium release and, because of the high spontaneous release over 48 hours (in excess of 50%), other viability assays were investigated.

^{111}Indium Release: An attempt was made to use ^{111}indium radiolabeled cells for cytotoxicity. As with ^{51}chromium, spontaneous release was very high over the 48 hour incubation period, making interpretation of xenobiotics difficult. Three experiments were performed using trichloroethylene and dexamethasone. ^{111}Indium release was only slightly elevated in the xenobiotic

FLOW CHART 2

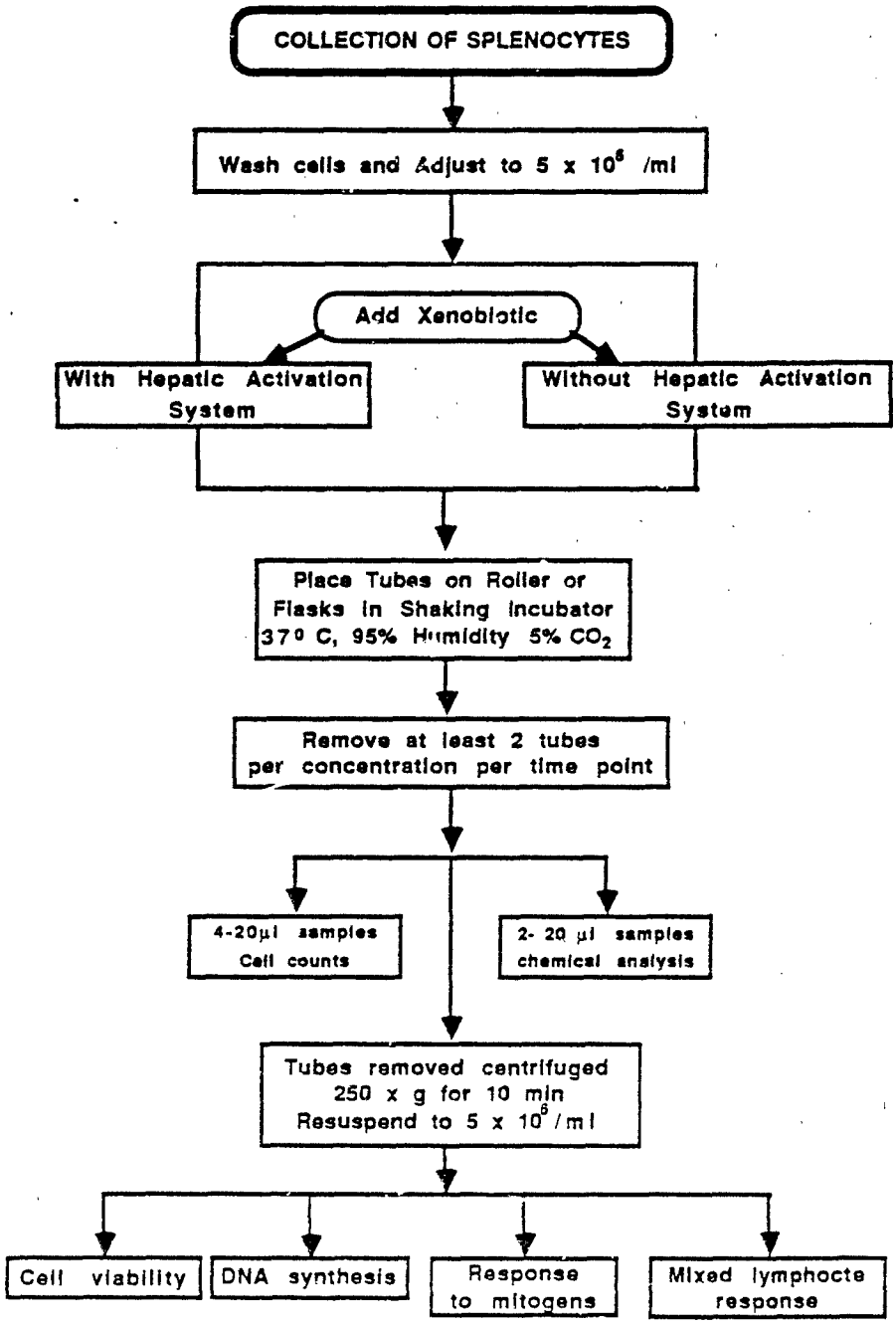
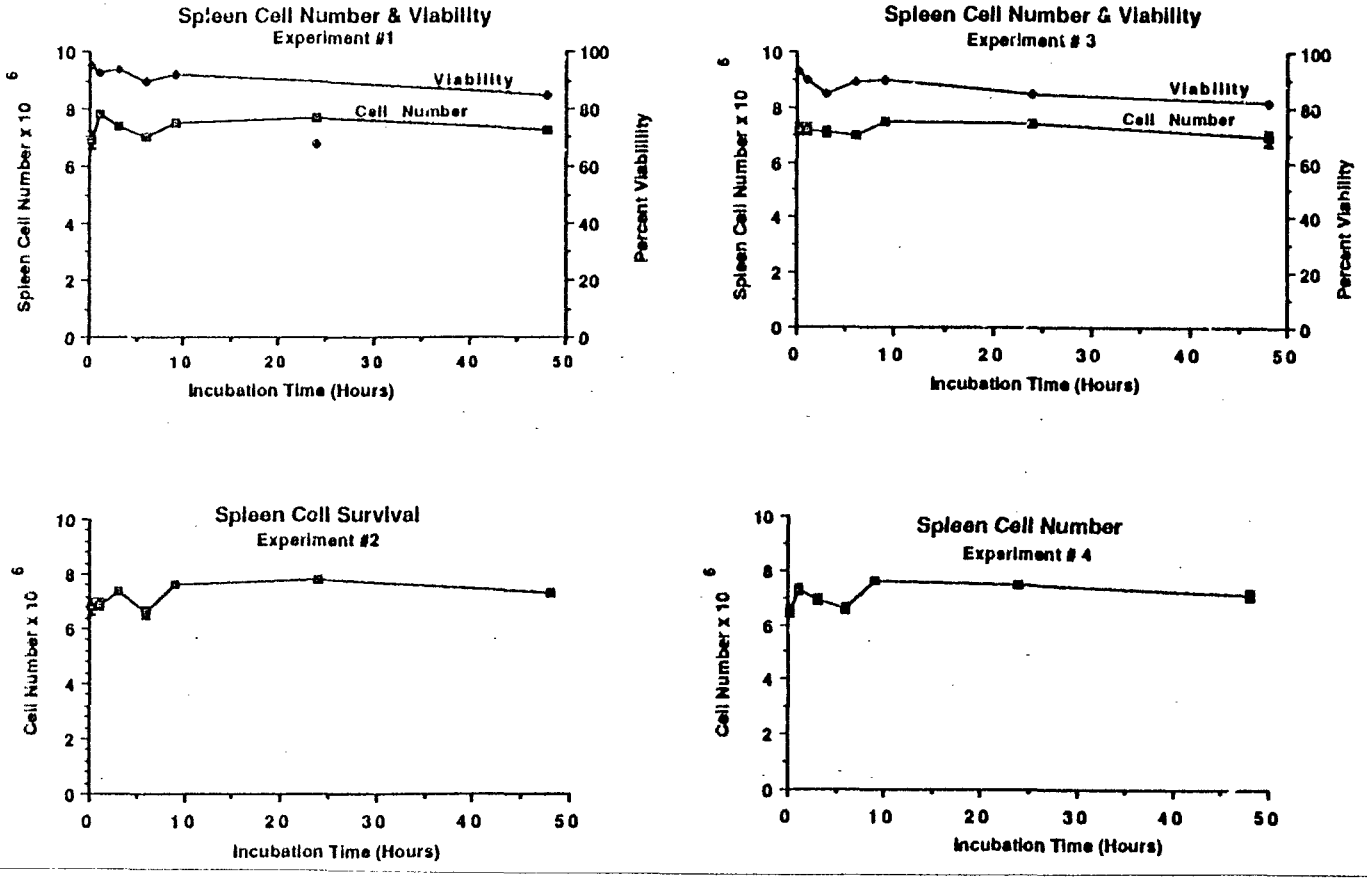

COLLECTION OF SPLENOCYTES
Wash cells and Adjust to 5 x 10⁶ /ml
Add Xenobiotic
With Hepatic Activation System
Without Hepatic Activation System
Place Tubes on Roller or Flasks in Shaking Incubator 37° C, 95% Humidity 5% CO₂
Remove at least 2 tubes per concentration per time point
4-20µl samples Cell counts
2- 20 µl samples chemical analysis
Tubes removed centrifuged 250 x g for 10 min Resuspend to 5 x 10⁶ / ml
Cell viability
DNA synthesis
Response to mitogens
Mixed lymphocte response

Figure 1

Spleen Cell Number and Viability over Forty Eight Hour Incubation Period



Spleen cells were incubated under standard conditions for 48 hours. At the indicated times, 3 culture tubes were removed and triplicate cell counts obtained on a Coulter Counter. The numbers represent the mean ± SE derived from the 3 culture tubes. Cell viability was performed by trypan blue exclusion. Four of 10 experiments are shown.

Table 1
Spleen Cell Number and Viability as a Function of Incubation Time

Incubation time (hrs)	cell number x 10^6 Exp. 1	Exp. 2	Exp. 3	Exp. 4
.25	6.9 ± 0.3 (95%)	6.8 ± 0.3	7.2 ± 0.2 (93%)	6.5 ± 0.2
1.0	7.8 ± 0.1 (93%)	6.9 ± 0.2	7.2 ± 0.2 (90%)	7.3 ± 0.2
3.0	7.4 ± 0.1 (94%)	7.4 ± 0.1	7.1 ± 0.2 (85%)	6.9 ± 0.2
6.0	7.0 ± 0.1 (90%)	6.6 ± 0.2	7.0 ± 0.1 (89%)	6.6 ± 0.2
9.0	7.5 ± 0.1 (92%)	7.6 ± 0.1	7.5 ± 0.1 (90%)	7.6 ± 0.1
24.0	7.7 ± 0.1 (90%)	7.8 ± 0.1	7.4 ± 0.1 (85%)	7.5 ± 0.1
48.0	7.3 ± 0.1 (85%)	7.3 ± 0.1	6.9 ± 0.3 (82%)	7.1 ± 0.2

Spleen cells were incubated under standard conditions for 48 hr. At the indicated times, 3 culture tubes were removed and triplicate cell counts obtained on a Coulter counter. The numbers represent the mean ± SE. The numbers in parentheses represent the cell viability as measured by trypan blue exclusion. Four of 10 experiments are shown.

exposed cultures over the appropriate vehicle cultures and no dose response could be obtained. Because of the high spontaneous release over 48 hours and the cost of the isotope, this approach was discontinued as a means of measuring cell viability.

Neutral Red Dye Uptake: In this assay, cells are exposed to neutral red dye, which will only be taken up by cells which are viable. This method was used in 15 experiments, and at times appeared to be more reproducible and sensitive than ^{51}chromium or ^{111}indium release. Over the course of the experiments, it became evident that the neutral red dye preparation changed and could not be reproducibly prepared in such a manner as to give consistent results. Because trypan blue exclusion and, eventually, the pronase method were more consistent, the neutral red dye uptake into viable cells was discontinued as a method for measuring cell viability.

Trypan Blue Exclusion: Cells were exposed to several xenobiotics. At various times following exposure, samples were removed and trypan blue dye was added. Cells were then examined under 100 x magnification and cells which took up dye were considered non-viable. Percent viability was determined by counting 200 cells. After two experiments using trichloroethylene and dexamethasone, it became evident that no significant change occurred in trypan blue exclusion when compared to vehicle controls, even in the presence of decreased cell number. After several additional experiments, it became apparent that the time between the ability of the cell to exclude the dye and cell lysis was short, that the dye exclusion assay was useful only during a short exposure period, and that at lower concentrations of a xenobiotic an equilibrium exists between the ability to exclude the dye and cell lysis.

Sensitivity to Pronase: Stewart et al. (1975) reported on the method presently being used to measure viability. The method takes advantage of the sensitivity of non-viable cells to pronase. Pronase is diluted to 3 mg/ml and 100 µl is added to 100 µl of cells in 13 ml polycarbonate tubes and incubated for 10 minutes at 37°C. Following the incubation, 10 ml of Isoton is added to the tubes and cell counts determined by Coulter counter. The percent viability is determined as by dividing the cell counts with pronase by the cell counts without pronase and multiplying by 100.

Spleen Cell DNA Synthesis

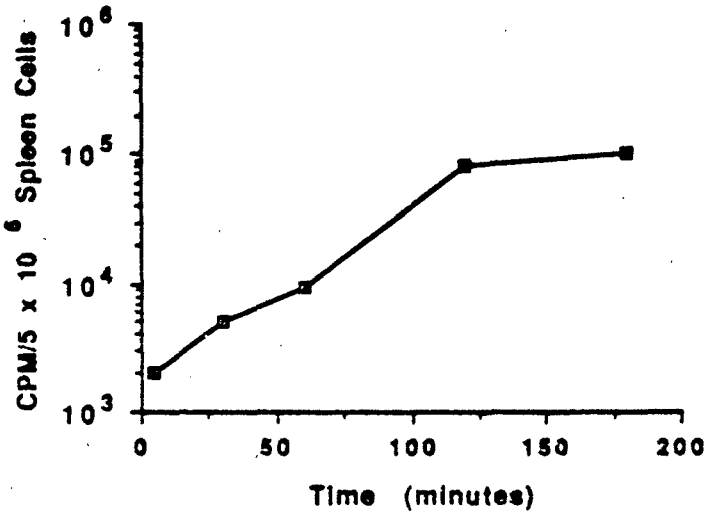
Spleen cells are continuously undergoing proliferation as they respond to internal and external stimuli. A good indicator of DNA synthesis is the incorporation of a DNA precursor into acid precipitable cellular material. For the earlier studies, ^{125}IUdR was used as the precursor because it was a gamma emitter and could be more quickly analyzed. Because of the increase in cost of ^{125}IUdR, studies now conducted use ^{3}H-thymidine. Appropriate studies were conducted and reported to show that both ^{125}IUdR and ^{3}H-thymidine are incorporated into acid precipitable material. The time course for incorporation of ^{125}IUdR into DNA of spleen cells is shown in Figure 2 and Table 2. The uptake is linear over the first two hours with a decreased rate of uptake from 2 to 3 hours. For most studies, uptake was at 1, 2, and 3 hours. For the time course studies, i.e., determining the effect of incubation time on spleen DNA synthesis, a 1 hour isotope pulse provides the best indicator of DNA synthesis.

DNA synthesis was measured over time in order to determine the stability of the system. The time course over 18 hours is shown in Figure 3 and Table 3. DNA synthesis is stable over 3 hours and then there is a sharp decline. Several variables, including cell density, amount and "lots" of fetal calf serum, and different media were investigated in order to maintain the DNA synthetic activity of spleen cells. None of the changes prolonged the DNA synthetic activity. Because cell viability does not change, the cells are most likely moving out of the S phase of the cell cycle.

Since DNA synthesis was determined based on a 1 hour pulse of isotope, a matrix study was conducted with incubation time as a variable and pulse time as a variable (Figure 4 and Table 4). There was no difference in the profile with a 1 hour pulse with no preincubation or 1 hour preincubation. Three hours of preincubation, coupled with a 1, 2, or 3 hour pulse period, showed decreased synthetic activity. Thus, the constraints for preincubation and pulse period are 3 and 1, respectively.

Figure 2

Time Course for Uptake of ^{125}IUdR



Spleen Cells were incubated with IUdR for 180 minutes in microtiter plates. Six wells wer analyzed for IUdR uptake at the indicated time periods. The numbers represent the mean ± SE derived from 6 samples for each time period.

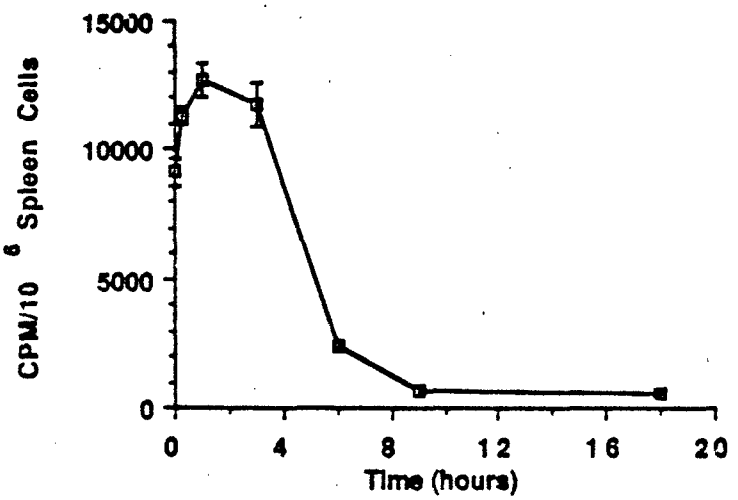
Table 2
Uptake of 125 IUdR into Spleen Cells

Incubation with 125 IUdR (minutes)	cpm/5 x 10^6 cells
5	2002 ± 50
30	5105 ± 407
60	9269 ± 500
120	10054 ± 602
180	100556 ± 789

Spleen cells were incubated with 125 IUdR for 180 minutes in microtiter plates. Six test wells were analyzed for 125 IUdR uptake at the indicated time periods. The numbers represent the mean ± SE derived from the six samples.

Figure 3

IUdR Incorporation Into Spleen Cells As a Function of Incubation Time



Spleen Cells were incubated under standard conditions for 18 hours. Three cultures were removed at the indicated time period and incorporation of IUdR was allowed to occur for 60 minutes. Numbers represent the mean ± SE derived from 3 tubes for each time period.

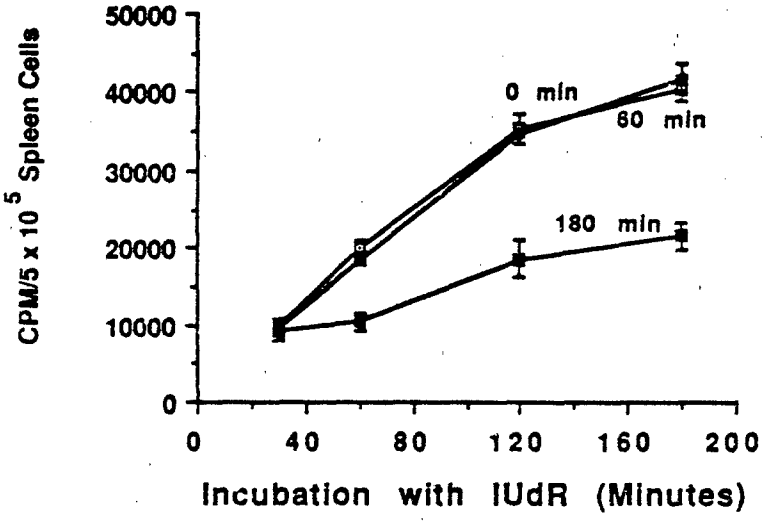
Table 3
Change in Incorporation of 125 IUdR into Spleen Cells as a Function of Incubation Time

Incubation without 125 IUdR (minutes)	CPM for 60 Minutes Incubation with 125 IUdR
0	9140 ± 535
15	11291 ± 345
60	12688 ± 668
180	11729 ± 859
360	2353 ± 120
540	660 ± 38
1080	560 ± 44

Spleen cells were incubated under standard conditions for 18 hr. Three culture tubes were removed at each indicated time period and incorporation of 125 IUdR was allowed to occur for 60 minutes. Numbers represent the mean ± SE derived from 3 tubes for each time point and 3 samples for each tube.

Figure 4

IUdR Incorporation Into Spleen Cells As a Function of Preincubation Time



Spleen cells were incubated under standard conditions for 0, 60, or 180 minutes prior to incubations with IUdR for 30, 60 or 180 minutes. The numbers repreent the mean ± SE derived from 3 culture tubes with 3 samples from each tube.

Table 4

Change in Incorporation of 125 IUdR into Spleen Cells as a Function of Preincubation Time

Incubation Time with 125 IUdR (min)	Incubation Time (minutes) Prior to Addition of 125 IUdR		
	0	60	180
30	9,776 ± 471	9,584 ± 381	9,354 ± 1317
60	20,141 ± 888	18,465 ± 531	10,397 ± 1140
120	35,437 ± 1974	34,730 ± 387	18,554 ± 2337
180	40,465 ± 1467	41,870 ± 1784	21,614 ± 1763

Spleen cells were incubated under standard conditions for 0, 60, or 180 minutes prior to incubation with 125 IUdR for 30, 60, 120, or 180 minutes. The numbers (CPM/5 x 10^5 cells) represent the mean ± SE derived from 3 culture tubes with 3 samples from each tube.

TIER 2

Spleen Cell Response to Mitogens

Spleen Cell Response to Concanavalin A and Bacterial Lipopolysaccharide

The response of spleen cells to the T cell mitogen, Concanavalin A, and the B cell mitogen, bacterial lipopolysaccharide, was characterized over a 48 hour period (Figures 5 and 6 and Table 5). Three concentrations of each mitogen were used and the optimum cell concentration and medium were determined in the previous studies. Responsiveness to mitogens is stable for 1 hour, decreases slightly by 3 hours, and decreases in a linear fasion to an unresponsive level by 48 hours. Figures 7 and 8 show data for the first 12 hours using Concanavalin A and LPS, respectively. This expands the decay part of the curve. As with DNA synthesis, changes in medium supplements, amounts and "lots" of fetal calf serum, 2-mercaptoethanol, and incubation gas mixtures did not alter the decay rate.

The efforts to prolong the time when spleen cells would be responsive to mitogens were important in order to provide the xenobiotic as much time as possible to bring about its potential effect. From these data, the longest period used for xenobiotic exposure was 3 hours.

In retrospect, because the cells are in single cell suspension, a xenobiotic is most likely to be able to enter the cell and produce its effect in a 3 hour period.

TIER 3

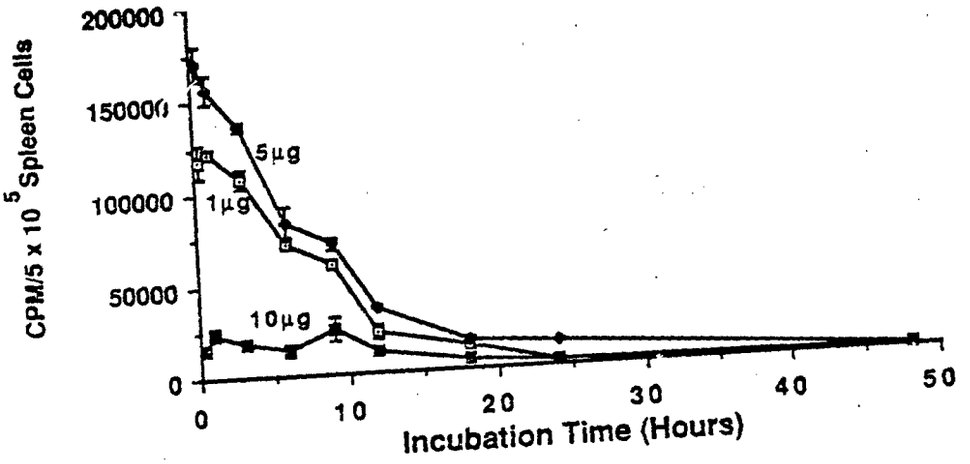
IgM Antibody Response to a T-Dependent Antigen

The T-dependent antibody forming cell system (Mishell-Dutton) has become the most powerful method of assessing the effects of xenobiotics on immunocompetent cells and determining the cellular target for the effect. Macrophages, T cells and B cells are all required for the successful production of an immunological product, the antibody.

The importance of this system and the impact it has made on toxicological studies related to the immune system has caused significant changes in the overall approach. This has become the first assay used and is combined with the cell number and the pronase method for viability.

Figure 5

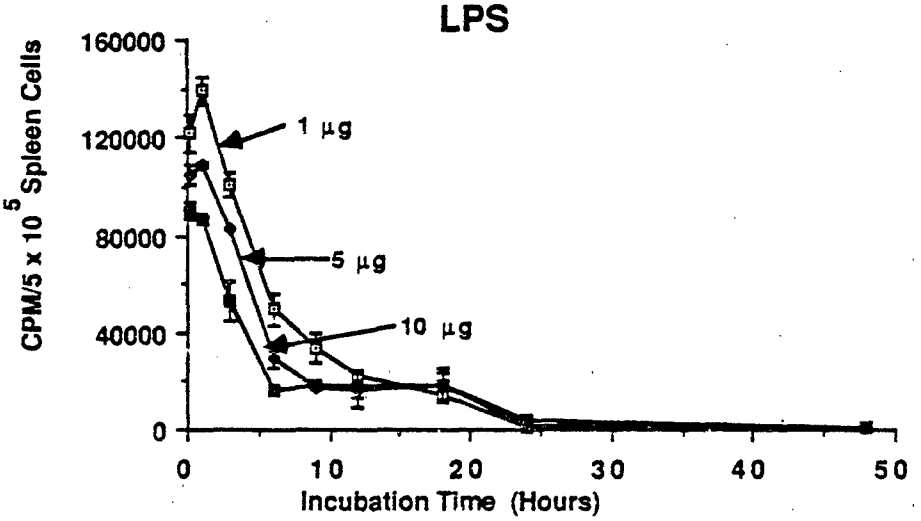
Decay of Spleen Cell Response
Concanavalin A



Spleen cells were incubated under the standard conditions. At the indicated times, 3 culture tubes were removed and 6 samples analyzed at each mitogen concentration. The numbers represent the mean ± SE of 3 tubes.

Figure 6

Decay of Spleen Cell Response
LPS



Spleen cells were incubated under the standard conditions. At the indicated times, 3 culture tubes were removed and 6 samples analyzed at each mitogen concentration. The numbers represent the mean ± SE of 3 tubes.

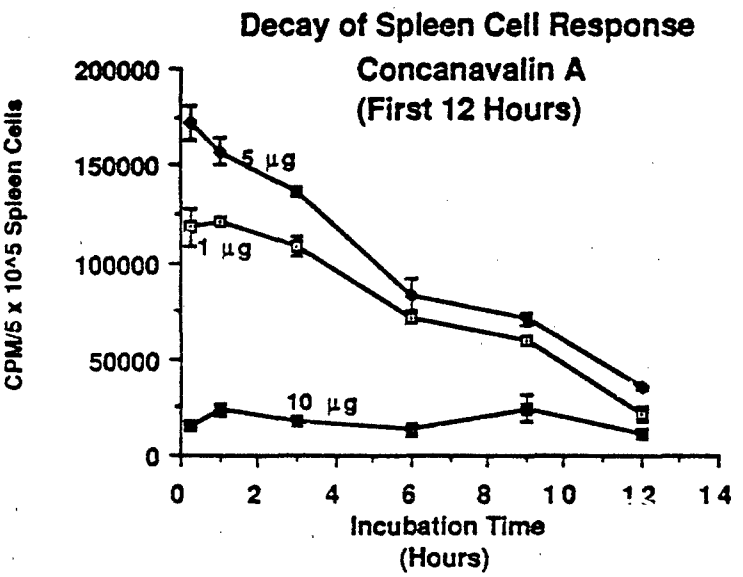
Table 5

Spleen Cell Response to Mitogens as a Function of Incubation Time

Incubation Time (hrs)	Con A (μg/5 x 10^5 cells) 1	5	10	LPS (μg/5 x 10^5 cells) 1	5	10
.25	117934 ± 9078	171481 ± 8861	15862 ± 1980	121997 ± 7442	105203 ± 3912	90167 ± 4016
1.0	121613 ± 358	157277 ± 7341	23808 ± 2986	139567 ± 5597	109044 ± 831	86211 ± 1366
3.0	108323 ± 5229	136419 ± 2720	18047 ± 2967	100865 ± 5348	82141 ± 81	52572 ± 8181
6.0	71024 ± 654	83268 ± 2458	13940 ± 3217	49483 ± 6961	29158 ± 3359	16252 ± 452
9.0	59521 ± 1226	71088 ± 8339	24744 ± 6546	33584 ± 5941	17796 ± 295	18083 ± 1391
12.0	21907 ± 3601	35358 ±b3249	11249 ± 1569	22042 ± 1261	16281 ± 6685	18760 ± 5216
18.0	12524 ± 1358	15868 ± 741	5120 ± 1647	14745 ± 4044	18061 ± 5392	18080 ± 7120
24.0	1987 ± 998	12422 ± 777	2287 ± 231	1430 ± 244	1660 ± 410	4369 ± 253
48.0	1620 ± 27	1436 ± 37	1467 ± 196	1508 ± 55	1470 ± 88	835 ± 35

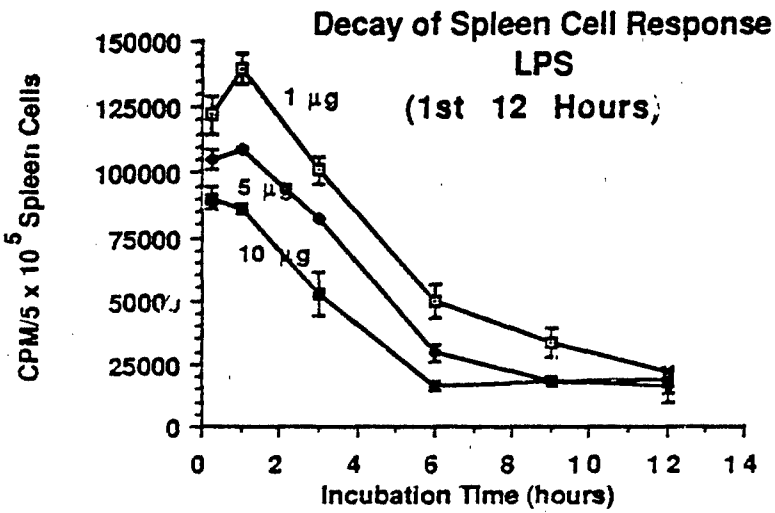
Spleen cells were prepared and incubated under standard conditions. At the indicated times, 3 culture tubes were removed and 6 samples analyzed at each mitogen concentration. The numbers (CPM/5 x 10^5 cells) represent the mean ± SE of the 3 tubes.

Figure 7



Spleen cells were incubated under the standard conditions. At the indicated times, 3 culture tubes were removed and 6 samples analyzed at each mitogen concentration. This is a subset of data from Figure 5 which expands the data over the first 12 hours. The numbers represent the mean ± SE of 3 tubes.

Figure 8



Spleen cells were incubated under the standard conditions. At the indicated times, 3 culture tubes were removed and 6 samples analyzed at each mitogen concentration. This is a subset of data from Figure 7 which expands the data over the first 12 hours. The numbers represent the mean ± SE of 3 tubes.

This system could not be successfully interfaced with the previously described format, i.e., exposure of cells in siliconized culture tubes, nor could the random bred mouse spleen cells be used. Pooling of spleen cells from random bred mice would lead to a mixed lymphocyte reaction that could produce cytotoxic or suppressor cells that would make interpretation difficult. Initially, BALB/c mice were used, but now the B6C3F1 female mouse is used. The B6C3F1 mouse is the NTP mouse of choice and is also used for the in vivo studies.

Mishell-Dutton Assay: Measurement of the in vitro IgM antibody response to sheep erythrocytes (Table 6) was conducted essentially as described by Mishell and Dutton (1967). The magnitude of the IgM response is dependent on the source of spleen cells, the presence of 2-mercaptoethanol and the matching of the sheep erythrocytes and the fetal calf serum. The basic assay (Figure 9) is as follows: Spleens are aseptically removed from mice and placed in a petri dish containing RPMI 1640 medium. Using toothed forceps, spleens are gently teased apart and the resulting cell suspension allowed to stand for 5 minutes. This permits larger debris to settle. The supernatant fluid is aspirated off and transferred to a sterile tube which is centrifuged at 900 xg for 10 minutes at 4°C and the supernatant is discarded. One ml of RPMI 1640 is added to each tube and the cells suspended. The cell concentration of each sample is determined and a portion of the cells is used to determine cell viability and a portion is used to measure the number of antibody forming cells. Presently, the best method for determining the antibody forming cell response is as follows: The matrix for the plaque assay consists of a 0.5% agar (Bacto-agar, Difco) solution in Essential Balanced Salt Solution (EBSS), heated to boiling in a microwave. Eight tenths of a milliliter of a 30 mg/ml solution of DEAE-Dextran(Pharmacia) in EBSS is added to 50 ml of agar. The agar solution is kept in a 37°C water bath and 0.35 ml is added to preheated 12 x 75 mm borosilicate tubes (Fisher), one tube per culture well. Sheep erythrocytes (30 µl of a 1:3 dilution in EBSS of the same sheep erythrocytes as were used for immunization) are added to the agar containing tubes. 50 µl of the spleen cell suspension is then added to the agar-sheep erythrocyte mixtures. At this point the tubes are removed from the water bath and 25 µl of cold 1:4 guinea pig complement (Gibco) in EBSS with Hepes is added and the tubes vortexed and poured into a 60 mm petri dish (Falcon) and covered with 50

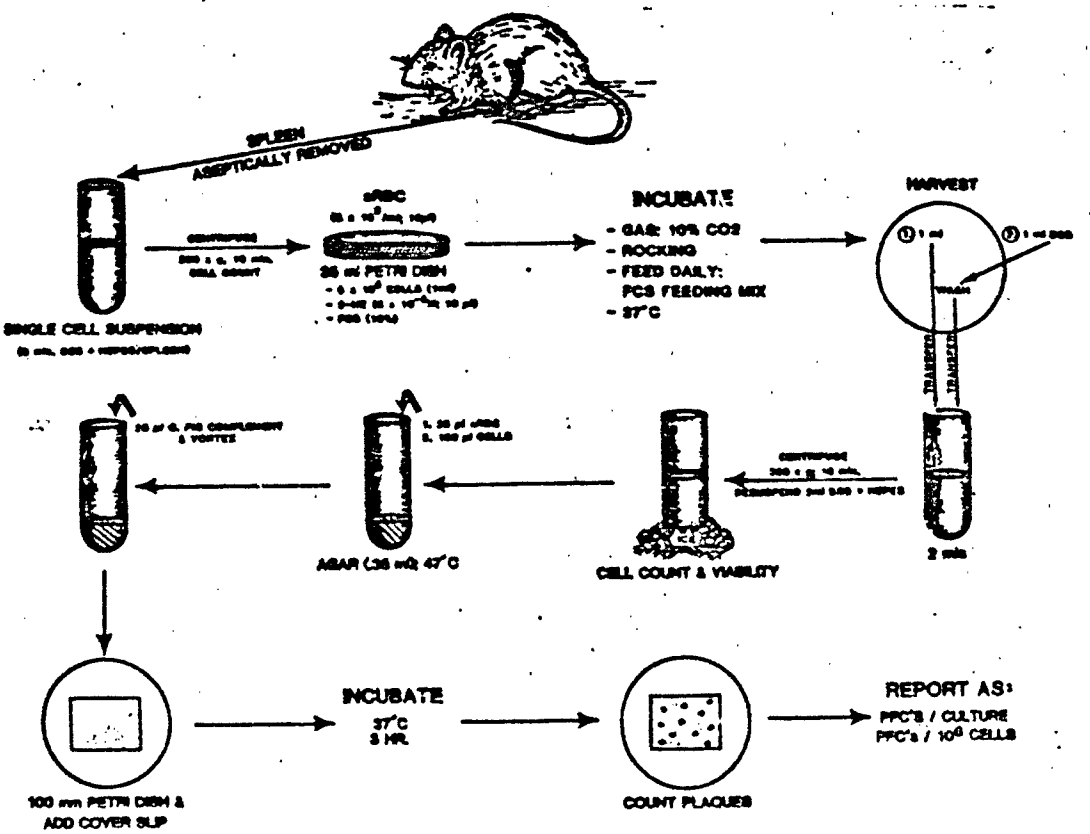
Table 6
Time Course for In Vitro Antibody Forming Cell Production

Day	Cells Recovered x 10^6	AFC/culture
3	5.6	165 ± 15
4	4.8	253 ± 37
5	5.5	801 ± 56
6	4.0	375 ± 74
7	3.2	275 ± 51
Background		22 ± 1

The standard Mishell-Dutton Assay was performed and the number of antibody-forming cells determined on days 3 - 7. The numbers represent the mean ± SE derived from 5 cultures per day.

FIGURE 9

MISHELL-DUTTON ASSAY



x 45 mm cover slips (Fisher) to obtain a monolayer. The plates are then incubated for 3 hours at 37°C, after which time the plaques are enumerated using a Bellco plaque viewer. The number of antibody forming cells (AFC) is enumerated and expressed as AFC/10^6 spleen cells or AFC/culture.

Figure 10 shows the cell number recovered and cell viability for the antibody forming cell response. Figure 11 shows the time course for the response. In contrast to the response to mitogens, spleen cells can be incubated for 24 hours without significant loss in the ability to respond to the sheep erythrocyte.

The basic culture system has been used with two other antigens which require different cell cooperation in order to produce antibody. The LPS used in this system is a polyclonal activator and DNP-Ficoll is a T-independent antigen. LPS is macrophage and T lymphocyte independent, while DNP-Ficoll is T-independent, requiring only macrophages and B cells to produce the response.

A complete set of data on the effects of a direct acting immunoactive drug (dexamethasone) in the Mishell-Dutton assay are shown in Table 7. This experiment illustrates the relationship among cell viability, time course of the response, and dose response. A graphic representation of the dose-response inhibition of the AFC by dexamethasone is shown in Figure 12.

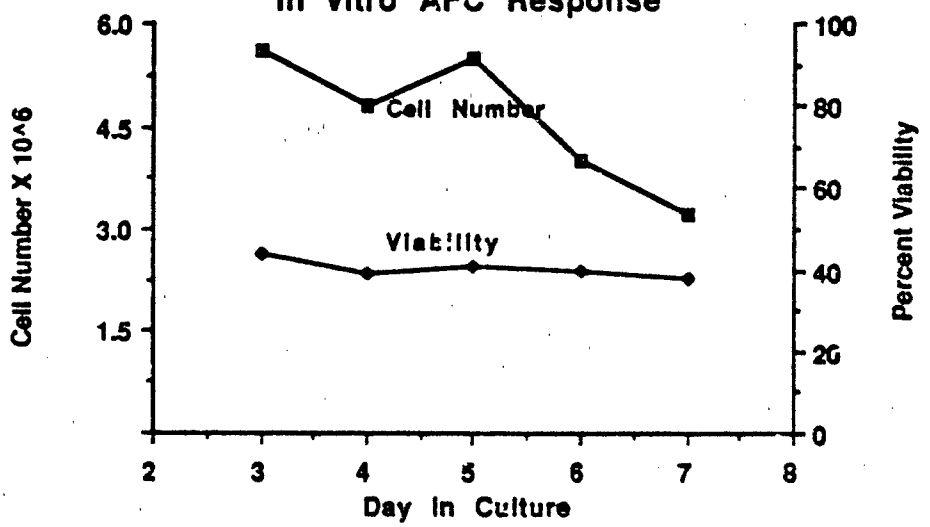
A second typical experiment using the Mishell-Dutton assay is shown in Table 8. Gallic acid produced a dose-dependent inhibition of AFC's, with the highest dose producing an inhibition slightly above the background level. The approximate ED50 is 1 µm.

Mixed Lymphocyte Reaction: The mixed lymphocyte reaction (MLR) is an in vitro assay for measurement of cell-mediated reactivity to specific cellular antigens coded by specific immune reactivity genes. This has been considered a Tier 3 assay because of the complexity of the cellular interactions. This assay measures the degree of proliferation induced by stimulator cells by measuring the incorporation of ^{125}IUdR into DNA of the responder cells.

Spleens are aseptically removed from CBA/H-Tb and DBA/2 mice and single cell suspensions prepared as described from the Mishell-Dutton assay. Mitomycin C is used to treat aliquots of each cell type. The treatment period is 30 minutes, followed by removal of the mitomycin C by washing with RPMI 1640 medium. The mitomycin-treated cells are used as stimulator cells. All cell concentrations are adjusted to 10^7 cells/ml and 50 µl aliquots dispensed

Figure 10

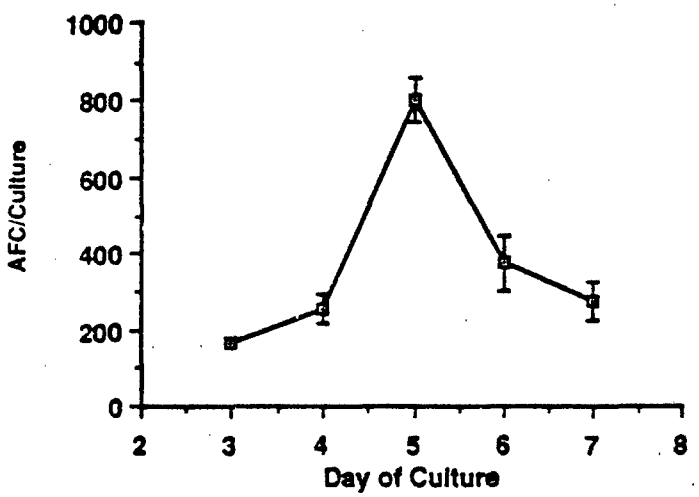
Cell Number & Viability
In vitro AFC Response



The in vitro antibody forming cell assay (Mishell Dutton) was performed and the number of recovered cells and viability were determined. The numbers represent the mean derived from 5 cultures per day. Viability was performed by trypan blue exclusion.

Figure 11

Time Course for IgM Antibody Forming Response



The in vitro antibody forming cell assay (Mishell Dutton) was performed and the number of antibody forming cells were determined. The numbers represent the mean derived from 5 cultures per day. Viability was performed by trypan blue exclusion.

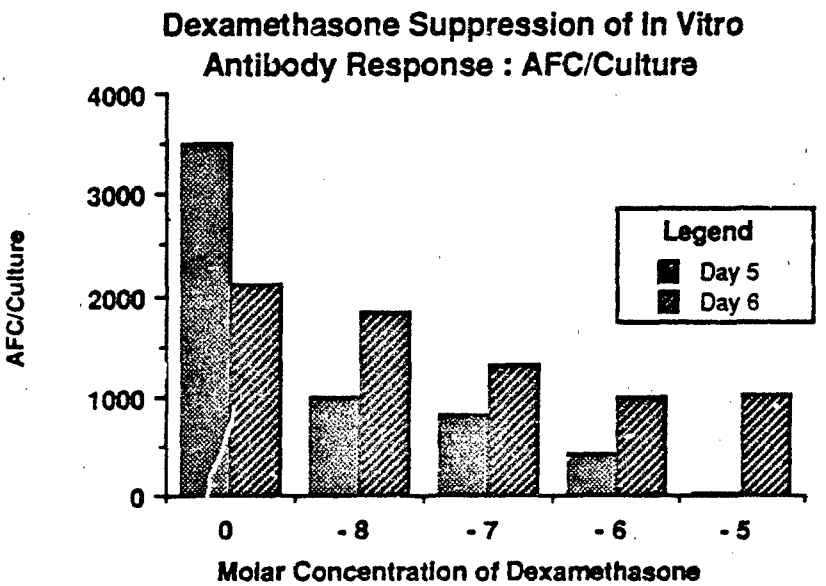
Table 7

Time Course of Dexamethasone Effects in the Mishell-Dutton Assay: Cell Recovery and Viability

Day	Background	Control	10^{-8}M	10^{-7}M	10^{-6}M	10^{-5}M
			Cell Counts (10^6 Cells)			
3	4.21 ± .26	4.42 ± .18	3.97 ± .27	3.85 ± .32	4.87 ± .06	3.28 ± .10*
4	3.06 ± .13	3.03 ± .22	1.26 ± .07*	1.64 ± .18*	1.59 ± .11*	1.81 ± .03*
5	4.14 ± .24	4.27 ± .43	1.14 ± .06*	1.07 ± .03*	1.33 ± .09*	1.00 ± .17*
6	2.88 ± .05*	3.51 ± .17	1.04 ± .06*	0.94 ± .08*	0.90 ± .08*	1.02 ± .07*
			Percent Viability (Trypan Blue)			
3	74 ± 4	72 ± 5	59 ± 4*	56 ± 3*	53 ± 2*	44 ± 4*
4	74 ± 5	85 ± 5	75 ± 5	58 ± 3*	61 ± 7*	52 ± 3*
5	76 ± 6	72 ± 3	86 ± 9	80 ± 5	67 ± 5	63 ± 4
6	77 ± 3	70 ± 5	87 ± 5*	85 ± 2*	80 ± 8	79 ± 6
			AFC per Culture			
3	70 ± 10*	348 ± 35	50 ± 10*	17 ± 7*	20 ± 6*	23 ± 3*
4	113 ± 15*	2954 ± 173	197 ± 93*	33 ± 3*	30 ± 6*	10 ± 6*
5	20 ± 12*	3542 ± 334	1180 ± 237*	574 ± 279*	460 ± 148*	10 ± 67*
6	10 ± 10*	2220 ± 200	1760 ± 114*	1310 ± 240*	910 ± 120*	1077 ± 67*
			AFC per 10^6 Cells			
3	17 ± 3*	79 ± 8	13 ± 3*	4 ± 2*	4 ± 1*	7 ± 1*
4	37 ± 5*	985 ± 58	151 ± 71*	21 ± 2*	19 ± 3.6*	6 ± 3*
5	5 ± 3*	824 ± 78	1073 ± 215	522 ± 253	354 ± 114*	10 ± 10*
6	3 ± 3*	634 ± 57	1692 ± 109	1394 ± 256*	1011 ± 134*	1056 ± 66*

The Mishell-Dutton Assay was set up using BALB/c female spleen cells. The cultures were exposed to dexamethasone at the indicated concentrations within 15 min of the sensitization of sheep erythrocytes. The assays were performed on days 3 through 6. Cell counts were performed on a Coulter counter. Trypan blue exclusion was performed for estimation of viability. Antibody forming cells were enumerated and expressed on a per 10^6 spleen cell and per culture basis. All numbers are expressed as mean ± SE derived from triplicate cultures. * = $P < .05$ based on a one way ANOVA and Dunnett's T test.

Figure 12



Spleen cells were exposed to Dexamethasone at the indicated concentrations under standard conditions. The bars represent the mean ± SE derived from triplicate cultures. Day 5 indicates that the cultures were harvested after 5 days. Similarly Day 6 indicates that the cultures were harvested after 6 days.

Table 8

Dose Response Using Gallic Acid in the Mishell-Dutton Assay

Gallic Acid	AFC/Culture	10^6 cells	AFC/10^6	Viability
None	2027 ± 106	4.03 ± .18	507 ± 33	35%
0.25 μM	2173 ± 158	3.89 ± .13	563 ± 48	33%
1 μM	1047 ± 107*	3.34 ± .07*	315 ± 34*	34%
4 μM	373 ± 64*	3.09 ± .12*	120 ± 20*	27%
16 μM	95 ± 37*	3.40 ± .13	28 ± 11*	27%

This experiment was carried out under standard conditions using spleens from male BALB/c mice and assayed on day 5. The values represent the means of 6 replicate plates. The background was 37 AFC/culture. Values differing from control at $p < .05$ using Duncan's multiple range test are noted by an asterisk.

into microtiter plate wells in the combinations shown in Figure 13.

Cultures are fed 10 µl of nutrient medium on days 2 through 7. Eighteen hours before harvest, 0.2 µCi of ^{125}IUdR and 50 λ of 4 x 10^{-6}M FUdR were added. Cells were harvested and counted on days 3 through 8. A typical control MLR response is seen in Figure 14 and the effect of the ethanol vehicle is seen in Figure 15.

Effects of Trichloroethylene: Trichloroethylene (TCE) was dissolved in 95% ethanol to yield 1.0, 0.1, 0.01 and 0.001M solutions. These were then added to aliquots of the cell suspensions to yield 1 x 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}M concentrations in the cultures. Vehicle controls received ethanol to produce a 1% concentration. Figure 16 shows the effect of TCE upon the mixed lymphocyte reaction. As previously reported, TCE at a concentration of 1 x 10^{-2}M suppresses lymphocyte blastogenesis, while other concentrations and the vehicle group are equal to or greater than control. However, trypan blue exclusion studies revealed that this effect is due to cytotoxicity rather than an effect upon the immune reaction itself.

Prior studies have indicated that TCE/ethanol may have a slight stimulatory effect upon the MLR. In this experiment, the vehicle group and 1 x 10^{-3}, 10^{-4}, and 10^{-5}M concentrations of TCE-2 showed modest, but significant, increases in ^{125}IUdR incorporation on days 6 and 7.

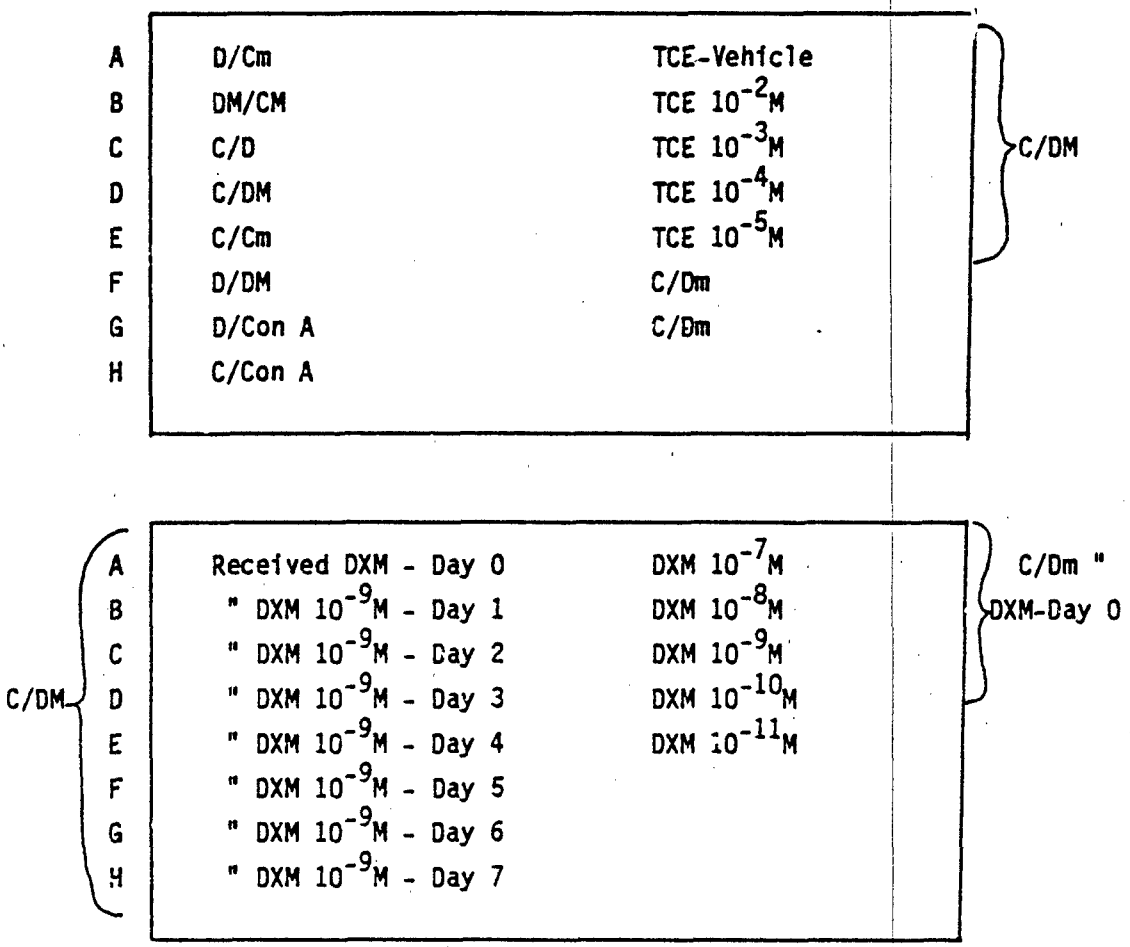
Effects of Dexamethasone: An additional experiment with temporal addition of dexamethasone to yield a 1 x 10^{9}M concentration in the cultures did not produce any unusual findings (Figure 17). Although there were a number of points of significant decrease in cpm, this may be due to normal variation within the experiment and does not represent a strong suppressive effect in our opinion.

The MLR assay may become a useful assay in this series; however, the expense allotted to animals and the time make it less attractive as a routine procedure.

Bone Marrow Parameters (Flowchart 3)

Bone Marrow Cell Number: The time course for changes in bone marrow cell number is shown in Table 9, and a representative experiment in Figure 18. The

Figure 13

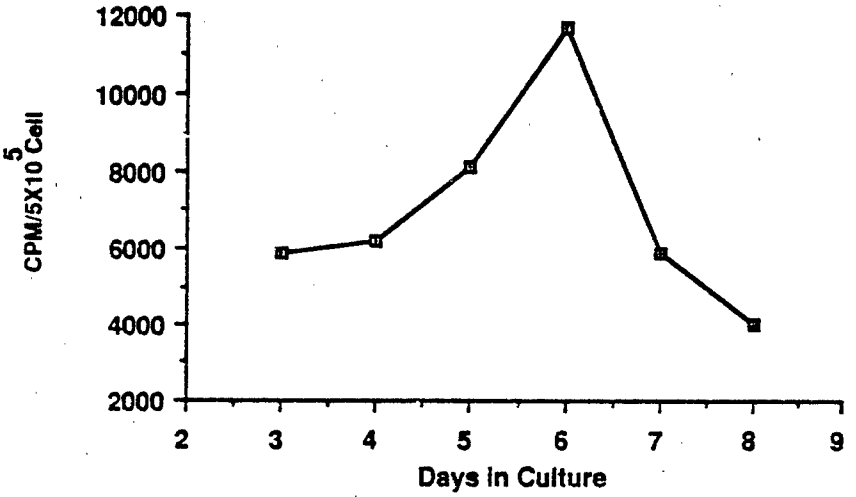


	Concentration	Viability
C -	.98 x 10^7/ml	87
Cm -	1.10 x 10^7/ml	87
D -	1.15 x 10^7/ml	82
Dm -	1.06 x 10^7/ml	89

Cell combinations and treatment for evaluation of the effects of TCE-2 and DXM upon the mixed lymphocyte reaction. The first plate shows routine controls as well as the dose-response curve for TCE-2. The second plate demonstrates the temporal addition and dose-response protocol. Below the plates are data giving the concentrations and percent viability of the different cell types from which the aliquots were taken at the beginning of the experiments. C = CBA spleen cells, D = DBA spleen cells, m = mitomycin treated. Cultures receiving Con A were given 5 µg/well.

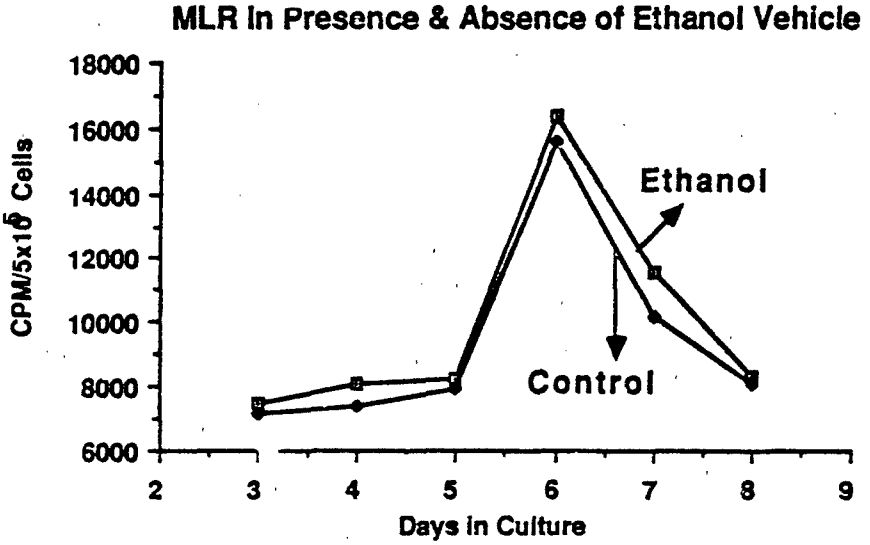
Figure 14

Time Course for MLR Response



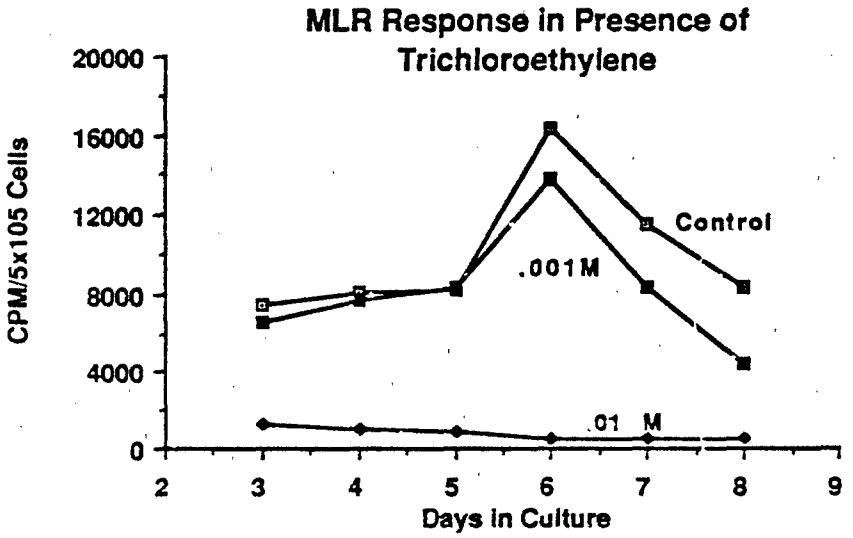
Mixed Lymphocyte Response: A typical control lymphocyte response (Dba/2 x C3H mice). Six replicates were assayed on each day in culture. The points represent the mean of 6 cultures. The standard error was within 10% of the mean.

Figure 15



Mixed lymphocyte response in the presence or absence of ethanol which is used as a vehicle for several xenobiotics. Each point represents the mean ± SE of 6 cultures wells.

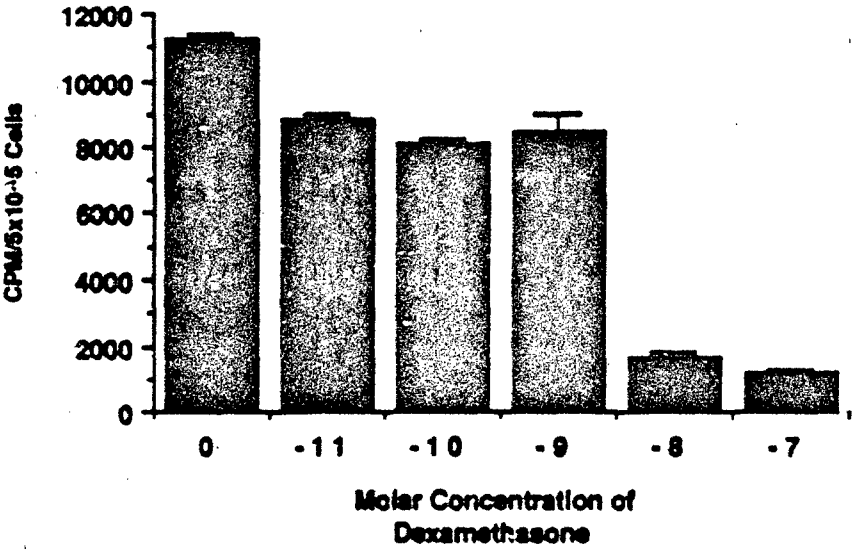
Figure 16



Mixed lymphocyte response in the presence and absence of .01M or .001 M Trichlorethylene. Six replicate cultures were harvested on the days of culture represented on the x axis. The points represent the mean of the 6 replicates. The standard error was within 10% of the mean.

Figure 17

Dexamethasone Suppression of MLR



Dexamethasone was added to the MLR cultures at the time of culture establishment. The peak response was on day 6 of culture. The bars represent the mean ±SE of six replicate culutes per concentration of dexamethasone.

FLOW CHART 3

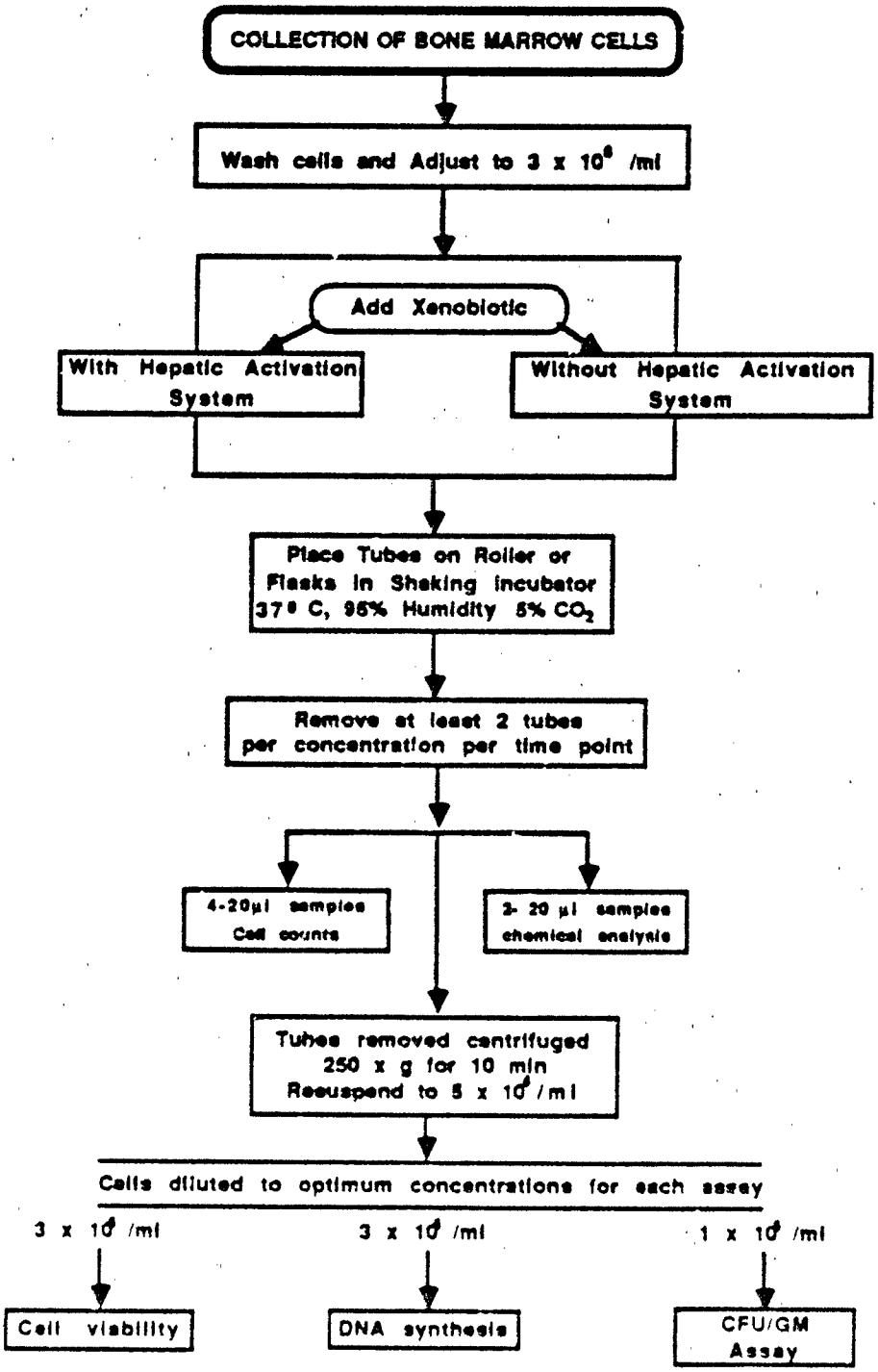

COLLECTION OF BONE MARROW CELLS
Wash cells and Adjust to 3 x 10⁶ /ml
Add Xenobiotic
With Hepatic Activation System
Without Hepatic Activation System
Place Tubes on Roller or Flasks in Shaking Incubator 37° C, 95% Humidity 5% CO2
Remove at least 2 tubes per concentration per time point
4-20µl samples Cell counts
3- 20 µl samples chemical analysis
Tubes removed centrifuged 250 x g for 10 min Resuspend to 5 x 10⁶ /ml
Cells diluted to optimum concentrations for each assay
3 x 10⁶ /ml
3 x 10⁶ /ml
1 x 10⁶ /ml
Cell viability
DNA synthesis
CFU/GM Assay

Table 9

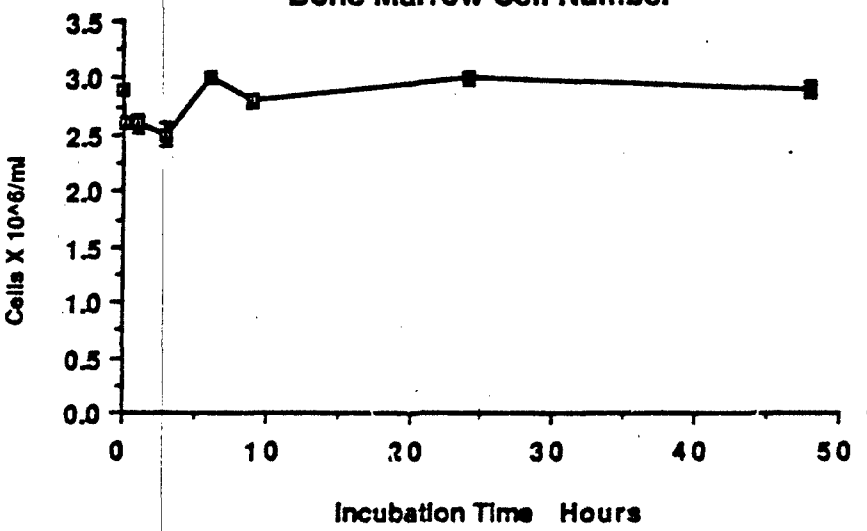
Bone Marrow Cell Number as a Function of Incubation Time

Incubation time (hrs)	cell number x 10^6/ml Experiment 1	Experiment 2	Experiment 3
0	3.1 ± 0.08	3.2 ± 0.06	2.9 ± 0.04
.25	2.3 ± 0.1	2.2 ± 0.05	2.6 ± 0.03
1	2.0 ± 0.04	2.5 ± 0.08	2.6 ± 0.07
3	2.1 ± 0.02	1.5 ± 0.30	2.5 ± 0.10
6	2.0 ± 0.04	2.3 ± 0.04	3.0 ± 0.03
9	2.2 ± 0.05	1.9 ± 0.06	2.8 ± 0.06
24	2.3 ± 0.07	2.3 ± 0.05	3.0 ± 0.05
48	2.0 ± 0.03	2.0 ± 0.04	2.9 ± 0.06

Bone marrow cells were incubated under standard conditions for 48 hours. At the indicated times, 3 cultures tubes were removed and triplicate cell counts obtained on a Coulter counter. The numbers represent the mean ± SE. Three of 10 experiments are shown.

Figure 18

Bone Marrow Cell Number



Bone marrow cells were incubated under standard conditions for 48 hours. At the indicated times, 3 culture tubes were removed and triplicate counts performed on the Coulter counter. The points represent the mean ± SE of the cell counts.

cell concentration was stable over the 48 hr incubation period. Cell viability studies in the bone marrow cells were highly variable because of the diversity of cells that take up trypan blue. In the specific studies using chemicals, trypan blue exclusion is shown.

DNA Synthesis: The time course for uptake of ^{125}IUdR into DNA of bone marrow cells is shown in Figure 19 and Table 10. Incorporation of ^{125}IUdR is linear over the 3 hour incubation period. As with the spleen cell preparation, the 1 hour incorporation period is used as the indicator of DNA synthesis.

DNA synthesis in bone marrow cells was measured over a 48 hour period. The results, shown in Figure 20 and Table 11, represent the 1 hour incubation with ^{125}IUdR and show stability over 1 hour, with a slight (30%) decrease after 3 hours and a sharp decline to minimal detectable activity at 24 hours. Manipulation of cell density, changes in medium, medium supplements, and fetal calf serum did not reproducibly prolong the time for which DNA synthesis could be maintained. The biggest variable as to the amount of incorporation of ^{125}IUdR was between bone marrow preparations.

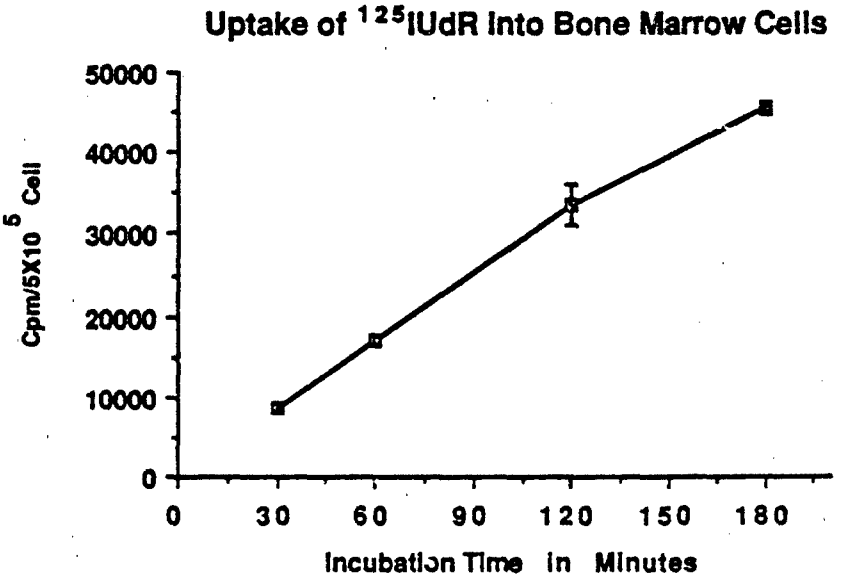
As with the spleen cells, we have set a limit of 3 hours for the exposure of the chemical prior to measuring DNA synthesis.

Bone Marrow Stem Cells - CFU-GM: Bone marrow stem cells have the longest survival when incubated under the standard conditions (data not shown). There was no significant change in stem cell number over a 48 hour incubation period.

HEPATIC MICROSOMAL ENZYME ACTIVATING SYSTEM

The successful employment of an *in vitro* toxicological test assay should include a hepatic mixed functional microsomal activating system that can allow for the *in vitro* conversion of a chemical to an active form or to its detoxification. Over the last 5 years we have developed a number of activation systems that can be interfaced with immunocompetent cells. The simplest system uses a 9000 xg liver supernatant from normal, phenobarbital and Arochlor 1254 induced livers. Additional induction methods have been used for specific substrates such as isopropanolol for the induction of DMN demethylase. The S9 fraction

Figure 19



Bone Marrow Cells were incubated with IUdR for 180 minutes in microtiter plates. Six test wells were analyzed for uptake at the indicated time period. Each point represents the mean ± SE derived from 6 samples.

Table 10

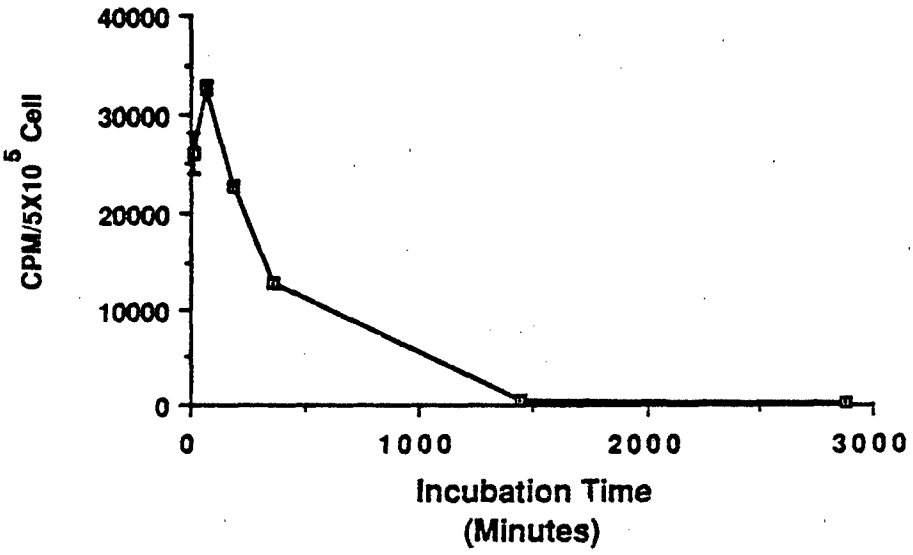
Uptake of 125 IUdR into Bone Marrow Cells

Incubation with 125 IUdR (minutes)	cpm/5 x 10^5 cells
30	8836 ± 396
60	17135 ± 649
120	33578 ± 2602
180	45502 ± 926

Bone marrow cells were incubated with 125 IUdR for 180 minutes in microtiter plates. Six test wells were analyzed for 125 IUdR uptake at the indicated time periods. The numbers represent the mean ± SE derived from 6 samples.

Figure 20

Decay of Incorporation of ^{125}IUdR Into Bone Marrow Cells



Bone marrow cells were incubated under standard conditions for 48 hours. Three culture tubes were removed at each indicated time period and incorporation of IUdR allowed to occur for 60 minutes. Points represent the mean ± SE of 3 tubes with 3 samples from each tube.

Table 11

Decay of Incorporation of 125 IUdR into Bone Marrow Cells as a Function of Incubation Time

Incubation without 125 IUdR (minutes)	CPM for 60 Minutes Incubation with 125 IUdR
15	26076 ± 2054
60	32637 ± 702
180	22866 ± 492
360	12866 ± 575
1440	571 ± 21
2880	321 ± 18

Bone marrow cells were incubated under standard conditions for 48 hours. Three culture tubes were removed at each indicated time period and incorporation of 125 IUdR allowed to occur for 60 minutes. Numbers represent the mean ± SE derived from 3 tubes for each time point and 3 samples for each tube.

can be maintained at -70°C for about 1 month without significant loss of activity. Purified microsomes have also been used in order to increase the activity/mg of protein. For the most part, the purified microsomes offer no advantage over the S9 fraction supernatant. The most complex system is the use of the primary hepatocyte as an activation system. This offers the optimal opportunity of xenobiotics to be activated or detoxified. The primary hepatocyte system requires greater technical skills and the assay system requires up to 12 hours to set up.

Preparation of S9 Fractions: The preparations that were successfully used included the 9000 x g liver supernatant fluid from normal, phenobarbital-induced and Arochlor 1254 induced mice. The mice used were BALB/c and CD-1.

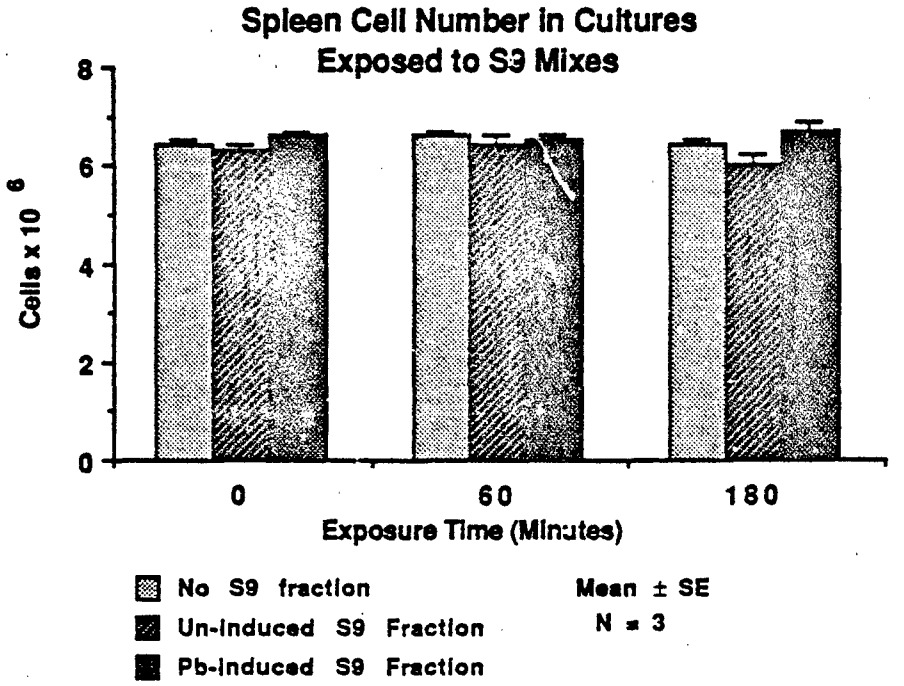
Phenobarbital was administered in the drinking water at 1 mg/ml for 1 week, with a 1 day withdrawal prior to the preparation of the S9 fractions. Arochlor 1254 was administered as a single intraperitoneal dose of 400 mg/kg. The S9 fraction was prepared 4 days later. The three preparations will be referred to as uninduced, phenobarbital-induced, and PCB-induced.

For preparation of the S9 fractions, the livers were removed aseptically and homogenized in 3 ml of cold sterile 1.15% KCl/g of liver. The homogenate was centrifuged at 9000 xg and the supernatant fluid removed and frozen in 1 ml aliquots. When used, a tube of the S9 was removed from -70°C storage, thawed, and added to a solution of cofactors. This "S9 mix" contained 3 mg S9 protein, 2 mM NADP, and 35 mM isocitrate in sterile culture medium, and was added directly to cell suspensions, 0.1 ml per ml of cell suspension.

Effects of S9 Mixes on the In Vitro Assays: The S9 mixes did not appreciably decrease cell number, cell viability, or response to the lymphocyte mitogen over 3 hours of exposure (Figures 21 and 22 and Tables 12-14). Two experiments are shown in Table 12. One experiment compared no S9 mix to the uninduced S9 mix, while the second experiment compares no S9 mix to the phenobarbital-induced S9 mix. Cell viability, as measured by trypan blue exclusion, was performed in the same studies, and the results (Table 13) show no adverse effects produced by the S9 mixes.

The expected decay of mitogen response over the 3 hr exposure period occurred (Figure 23). However, there was no S9 mix effect on either the T cell response to Concanavalin A or the B cell response to bacterial lipopolysac-

Figure 21


Spleen Cell Number in Cultures
Exposed to S9 Mixes
Cells x 10 6
8
6
4
2
0
0
60
180
Exposure Time (Minutes)
No S9 fraction
Un-induced S9 Fraction
Pb-induced S9 Fraction
Mean ± SE
N = 3

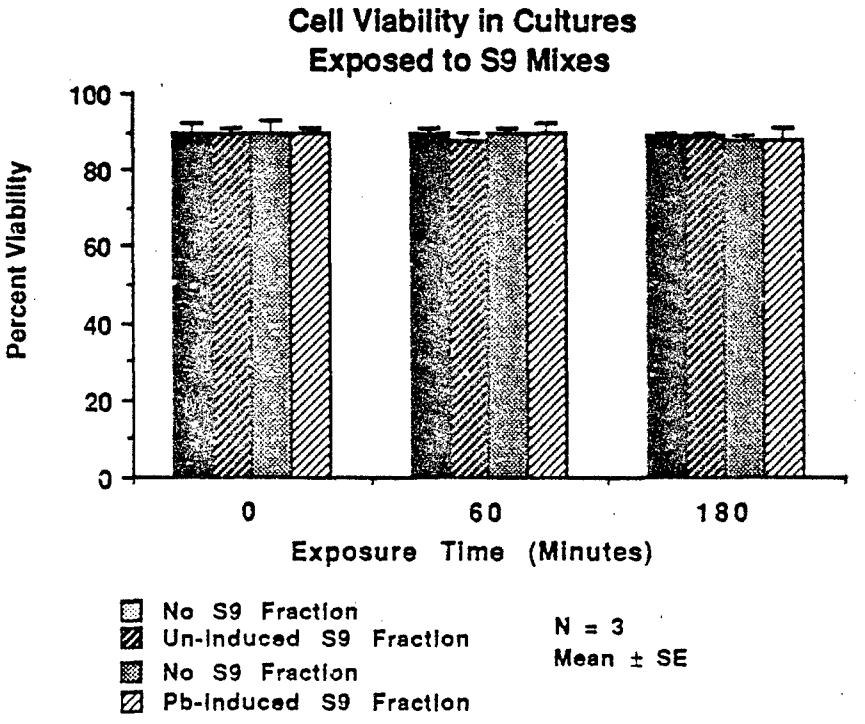
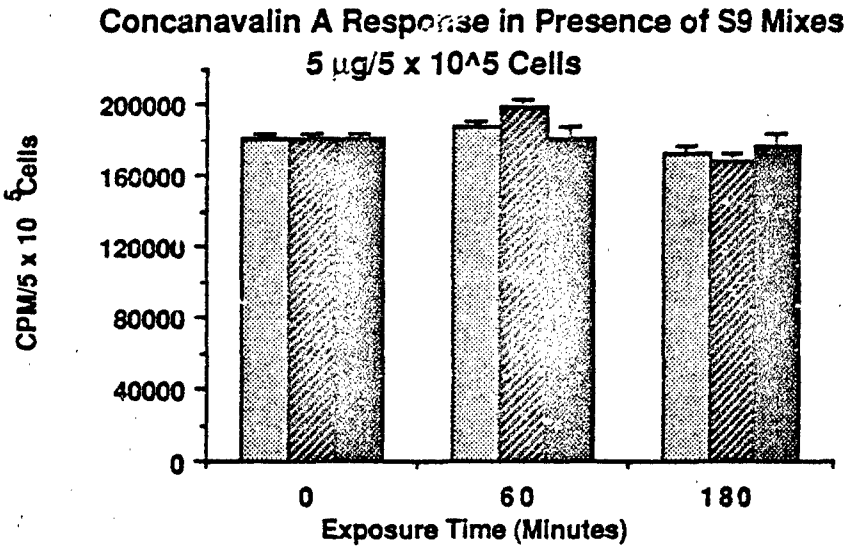

Cell Viability in Cultures
Exposed to S9 Mixes
Percent Viability
100
80
60
40
20
0
0
60
180
Exposure Time (Minutes)
No S9 Fraction
Un-induced S9 Fraction
No S9 Fraction
Pb-induced S9 Fraction
N = 3
Mean ± SE

Figure 22


Concanavalin A Response in Presence of S9 Mixes
5 µg/5 x 10^5 Cells
CPM/5 x 10 5Cells
200000
160000
120000
80000
40000
0
0
60
180
Exposure Time (Minutes)

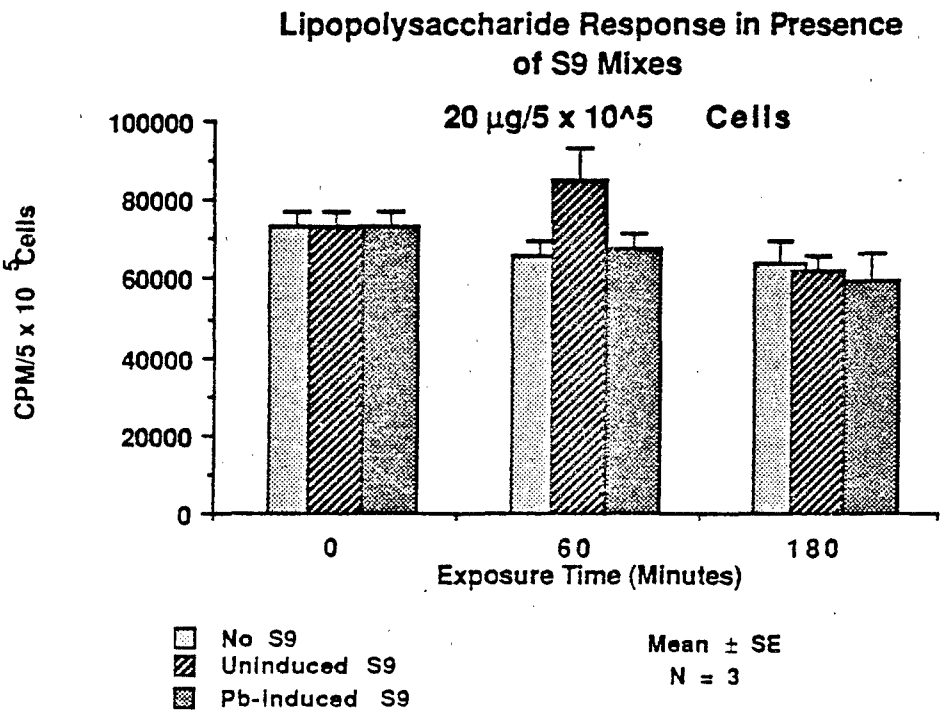

Lipopolysaccharide Response in Presence of S9 Mixes
20 µg/5 x 10^5 Cells
CPM/5 x 10 5Cells
100000
80000
60000
40000
20000
0
0
60
180
Exposure Time (Minutes)
No S9
Uninduced S9
Pb-Induced S9
Mean ± SE
N = 3

Table 12

Spleen Cell Number in Cultures Exposed to S9 Mixes

	Exposure Time (minutes)		
	0	60	180
No S9	6.4 ± 0.1	6.6 ± 0.1	6.4 ± 0.1
Uninduced S9	-	6.4 ± 0.2	6.0 ± 0.2
No S9	6.8 ± 0.1	7.2 ± 0.2	6.9 ± 0.2
PB Induced S9	-	6.5 ± 0.1	6.7 ± 0.2

Spleen cell suspensions obtained from CD-1 male mice were exposed to uninduced or phenobarbital (PB) induced S9 mixes for 3 hours. Cell counts were determined from 3 culture tubes at the indicated times. Triplicate cell counts were taken on each tube. The numbers represent the mean ± SE derived from the 3 tubes.

Table 13

Cell Viability in Cultures Exposed tc S9 Mixes

	Exposure Time (minutes)		
	0	60	180
No S9	90 ± 2	90 ± 1	89 ± 1
Uninduced S9	-	88 ± 2	89 ± 1
No S9	90 ± 3	90 ± 1	84 ± 1
PB Induced S9	-	90 ± 2	88 ± 3

Spleen cell suspensions obtained from CD-1 male mice were exposed to uninduced or phenobarbital (PB) induced S9 mixes for 3 hours. Cell viability was determined by trypan blue exclusion from 3 culture tubes at the indicated times. Triplicate viability tests were performed on each tube. Numbers represent the mean ± SE derived from 3 tubes.

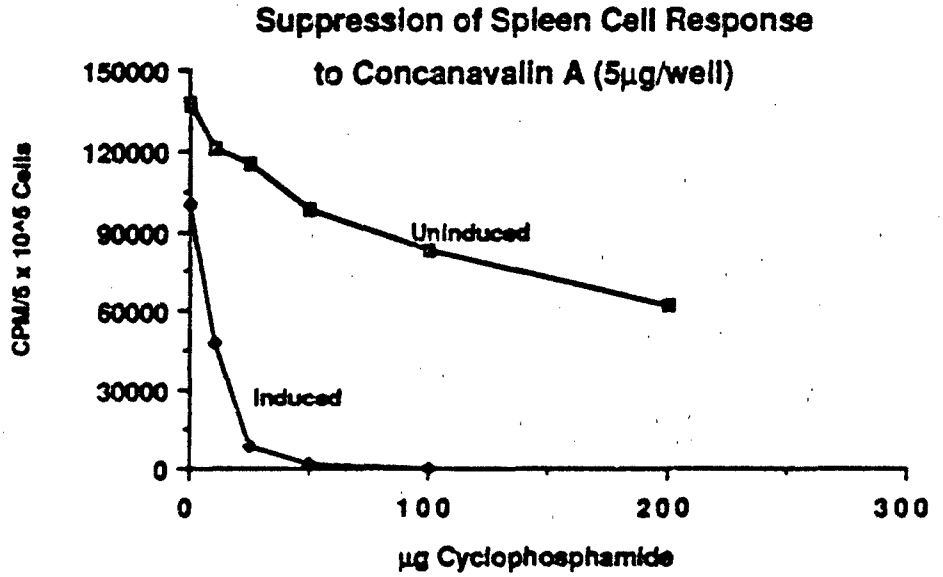
Table 14

Spleen Cell Response to Optimum Concentrations of Concanavalin A and Bacterial Lipopolysaccharide

	Exposure Time (minutes) 0	60	180
	Concanavalin A 5 µg/5 x 10^5 spleen cells		
No S9	181900 ± 1970	188411 ± 2715	173225 ± 3958
Uninduced S9		198808 ± 4939	168441 ± 4301
PB Induced S9		181017 ± 6601	177331 ± 6102
	Bacterial Lipopolysaccharide (20 µg/5 x 10^5 spleen cells		
No S9	73315 ± 3931	65988 ± 3590	64124 ± 5410
Uninduced S9		85246 ± 7778	62368 ± 3601
PB Induced S9		67418 ± 3882	59571 ± 6810

Spleen cell suspensions obtained from CD-1 male mice were exposed to uninduced or phenobarbital (PB) induced S9 mixes for 3 hours. At the indicated times, cells from each of 3 culture tubes were added to microtiter plates containing the mitogen. Responsiveness was measured 72 hours later. The numbers represent cpm/5 x 10^5 spleen cells and are the mean ± SE derived from the 3 culture tubes.

Figure 23



Comparison of uninduced and induced S9 preparations. Cyclophosphamide was incubated in the presence of S9 preparation and spleen cells for 1 hour. The cells were washed and the spleen cell response to Concanavalin A was measured. The points represent the mean of 3 culture tubes, with 4 replicates for each tube. Induced S9 was prepared from phenobarbital treated mice.

charide (Table 14). Only the optimal concentrations of mitogen are shown. At the sub and superoptimal concentrations, there was no S9 mix associated alteration in response.

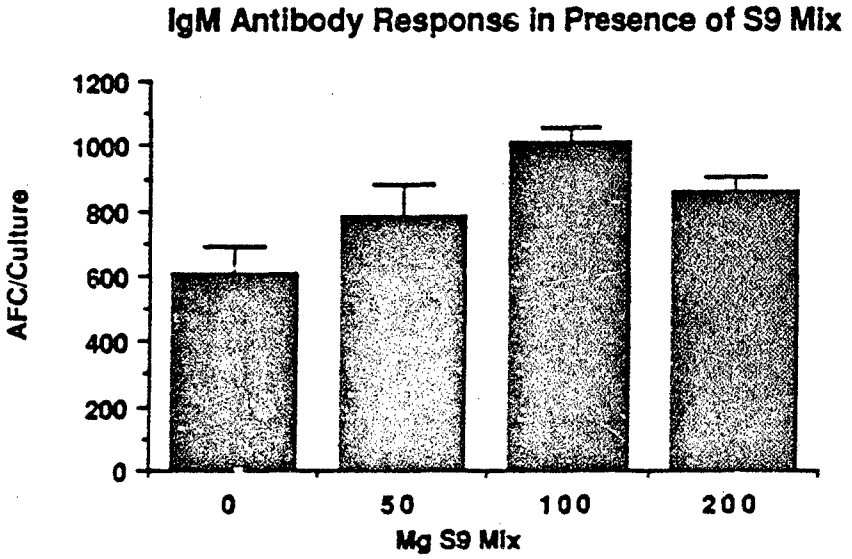
The S9 mixes were successfully interfaced with the Mishell-Dutton assay (Figure 24). Several experiments were performed to determine the amount of S9 protein, the type of preparation, i.e., purified microsomes vs 9000 xg liver supernatant along with cofactors. Table 15 illustrates one experiment, showing that 0.4 mg and above of microsomal protein was toxic. Additionally, the cofactor mix alone was toxic, which was found to be the isocitrate dehydrogenase. However, the cofactors and the S9 fraction together did not reduce the number of cells recovered or the number of antibody producing cells (Tables 16 and 17). The amount of S9, up to 200 mg/culture, did not produce a toxic effect with 1 hour exposure (Table 16). The incubation time with the S9 mix longer than 1 hour causes diminution of the antibody forming cell response. Thus, the time period for allowing a chemical to become metabolized in the presence of spleen cells in the Mishell-Dutton assay is 1 hour.

The S9 mixes were capable of carrying out at least one of the oxidation reactions, as seen in the conversion of cyclophosphamide to its active form. An example of this is seen in the ability of cyclophosphamide to inhibit the lymphocyte response to mitogens and to inhibit the development of antibody-forming cells in the Mishell-Dutton assay. The data seen in Table 18 and Figure 25 shows the dose-dependent reduction in the spleen cell response to the optimal concentrations of Concanavalin A and bacterial lipopolysaccharide.

BASELINE AND ED50 VALUES FOR POSITIVE CONTROLS

Although additional refinements are underway for the development of the tier assay systems in both the spleen and the bone marrow, certain chemicals have been used as positive controls and certain baseline values can be expected when the cells are cultured under the conditions that we have described. For looking at cell viability, we have chosen trichloroethylene because of the number of studies that have been conducted using it. Invariably, 10^{-2}M or 1340 µg/culture kills about 50% of the spleen cells, as

Figure 24



Cultures of spleen cells were exposed to phenobarbital induced S9 mix for 60 minutes prior to washing of spleen cells and iinmunizing with sheep erythrocytes. The in vitro antibody forming cell response was performed on day 5 which was peak response day. Each column represents the mean ± SE derived from 5 cultures.

Table 15

Effects of Microsomal Protein and NADPH on the Mishell-Dutton System

	Culture Conditions	AFC/Culture
I.	Background	120 ± 57
	Control	628 ± 153
	0.6 mg S9 protein	none detected
	0.2 mg microsomal protein	852 ± 53
	0.4 mg microsomal protein	475 ± 148
	0.6 mg microsomal protein	120 ± 28
	0.8 mg microsomal protein	none detected
	Control, 1 hr incubation	1005 ± 35
	0.5 mg S9, 1 hr incubation	758 ± 11
	Cofactor mix	none detected
II.	Background	147 ± 37
	Control	1493 ± 191
	Isocitrate + NADP (0.1 mM)	1220 ± 318
	Isocitrate dehydrogenase	493 ± 87
	NADPH (0.04 mM)	1927 ± 291
III.	Background, 1 hr incubation	240 ± 70
	Control, 1 hr incubation	1627 ± 113
	S-9 mix, 1 hr incubation	1087 ± 316

Spleens were obtained from female BALB/c mice, cultured under standard conditions, and assayed on Day 4. S9 refers to the 9000 xg supernatant from a fresh KCl mouse liver homogenate. Microsomes were collected from a portion of the S9 by centrifugation at 100,000 xg. For the samples where "1 hr incubation" is indicated, the cultures were removed from the incubator after 1 hr, centrifuged, and the cells put into fresh culture medium and returned to the incubator. The complete cofactor mix consists of 40U isocitrate dehydrogenase, 32 mg NADP, and 180 mg isocitrate in 20 ml KCl. Fifty µl of this is added to each culture. When the components are added singly, the same amounts are used. In part III, "S9 Mix" is 35 mM isocitrate, 2 mM NADP, and 5 mg S9 protein per ml. The S9 protein was from phenobarbital induced mouse liver and had been stored at -70°C. Fifty µl of this was added to each culture.

Table 16

Response of the Mishell-Dutton Assay in the Presence of S9

Mg S9 Mix Per Culture	Cells x 10^6 Per Culture	AFC/Culture
0	3.4 ± 0.4	610 ± 80
50	3.6 ± 0.1	786 ± 92
100	3.5 ± 0.1	1011 ± 42
200	3.3 ± 0.2	860 ± 47

PB induced S9 mix was incubated with spleen cells from BALB/c mice for one hour. The cells were then washed in fresh medium and sheep erythrocyte antigen added. The number of recovered cells and antibody-forming cells (AFC) were determined on day 5 of culture. The numbers represent the mean ± SE derived from triplicate cultures.

Table 17

Antibody-Forming Cell Response (Mishell-Dutton)
in Cultures Exposed to S9 Mixes

	Recovered cells x 10^6	AFC/Culture	AFC/10^6 Cells
No S9	5.6 ± 0.5	1760 ± 210	317
Uninduced S9	5.0 ± 0.2	2520 ± 218	504
PB Induced S9	5.1 ± 0.4	2227 ± 154	441

Spleens obtained from BALB/c mice were exposed to three different preparations of S9 mixes for one hour. The cells were washed and sheep erythrocyte antigen added. The AFC and recoverable spleen cells were enumerated on day 5 of culture. The numbers represent the mean ± SE derived from 3 cultures. The background plaques/culture was 103 ± 23.

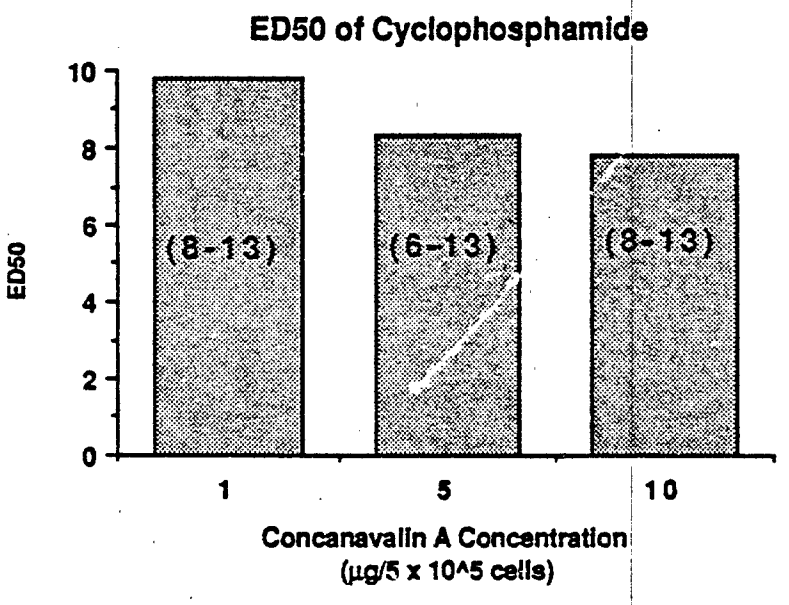
Table 18

In Vitro Inhibition of Antibody-Forming Cell Response (Mishell-Dutton) by Cyclophosphamide

	AFC/Culture	cells x 10^6 per culture
No S9 Mix	2520 ± 218	5.0 ± 0.2
Uninduced S9 Mix	807 ± 140	4.1 ± 0.6
PB Induced S9 Mix	0	1.9 ± 0.2

Spleen cells and the S9 mixes were incubated for 1 hour with 2.6 µg/culture of cyclophosphamide. The cells were then washed with fresh medium and the culture initiated with the addition of sheep erythrocyte antigen. The numbers represent the mean ± SE derived from 3 cultures.

Figure 25



ED50 of Cyclophosphamide. Spleen cells were incubated with cyclophosphamide and phenobarbital-induced S9 preparation for 1 hour prior to measurement of mitogen induced response. The bars represent the ED50, and the numbers in the bars represent the 95% confidence limits.

measured by trypan blue exclusion. However, the actual cell number decrease can be seen with an approximate ED50 of 600 μg/ml. This difference between viability and cell number is a function of the disintegration of these cells shortly after they lose their ability to exclude trypan blue. The baseline viability of the spleen cells throughout the study was between 78-98%.

Baseline ED50 values of the positive controls for DNA synthesis were as follows: After 60 minutes of incubation with ^{125}IUdR the range of cpm/5 x 10^6 cells was between 4100 and 15,000. We have had cpm's at much higher levels, but we believe that this was because there was some infectious process going on the mouse. The ED50 value for the positive control cytosine arabinoside was 11 μg with 95% confidence limits of 3 to 25 μg. Cyclophosphamide is able to inhibit DNA synthesis with an ED50 of 200 μg per culture. This effect is seen only when cyclophosphamide is administered with a phenobarbital-induced S9 mix. Even though we are calling cyclophosphamide a positive control in this system, it is the only positive control that we have that works with phenobarbital-induced S9 mix.

The normal and ED50 values of the positive controls were the spleen cell response to mitogens, as shown in Table 19. The baseline responses to the mitogens are shown and are a function of the amount of mitogen. With Con A, there is a bell-shaped curve, which is well-known. In the case of LPS, the response is about the same, regardless of the concentration of mitogen used. These ranges are taken from 28 experiments that have been conducted under the standard conditions. The positive control in this system is dexamethasone in the absence of an S9 mix. The ED50's are the smallest of any of the compounds tested. Approximately 1 μg/5 x 10^6 cells will give an ED50 for this response. In a screening mode, one would not use all concentrations of the mitogens, but could use two concentrations. Cyclophosphamide was very active in this system in the presence of the S9 mix, with ED50 values ranging between 3 and 8 μg/5 x 10^6 cells. This is a very reproducible effect and represents a good positive control.

The baseline and ED50 values for the positive controls for Mishell-Dutton assay have been determined. The antibody forming cell baseline response should be between 1000 and 4000 AFC/culture. Viability on the peak day of response should be between 30 and 40%, and dexamethasone should produce an ED50 of about 5 μg/culture. Again, cyclophosphamide represents a very good positive control in the presence of an induced S9 mix, with an ED50 of approximately 6 μg/

Table 19

Normal and ED50 Values of Positive Controls
for Spleen Cell Response to Mitogens

	Baseline Response	Dexamethasone ED50	Cyclophosphamide ED50
Con A 1 µg	30,000 - 180,000	2.5 (0.3 - 7.5)	3 (1 - 15)
5 µg	140,000 - 200,000	1.9 (1.0 - 3.5)	8 (4 - 9)
10 µg	40,000 - 160,000	0.7 (0.6 - 3.0)	8 (3 - 12)
LPS 5 µg	50,000 - 70,000	0.9 (0.6 - 4.0)	6 (3 - 10)
10 µg	20,000 - 70,000	0.7 (0.6 - 1.8)	7 (3 - 11)
20 µg	20,000 - 70,000	0.7 (0.6 - 1.8)	8 (2 - 10)
40 µg	15,000 - 60,000	0.8 (0.6 - 1.9)	8 (3 - 12)
No Mitogen	3,500 - 7,000	-	-

Spleen cells were exposed to dexamethasone and cyclophosphamide under standard conditions. The baseline response is expressed as cpm per 5×10^5 cells. The ED50 is in µg based on 5×10^6 cells, with 95% confidence limits in parentheses.

culture.

The mixed lymphocyte reaction has more variability than the other assays. The baseline response measured on peak day should be 7,000 to 20,000, and the viability on peak day should be 20 to 40%. The two positive controls that can be used in this system are dexamethasone and cyclophosphamide. Good definitive ED50's have not been established; however, in four experiments it appears that the ED50 for dexamethasone should be about 5 μg/culture (5×10^5 responder cells). Cyclophosphamide is a good control for the presence of the S9 mix and in our hands we have found the ED50 to be about 15 μg/culture.

For the bone marrow cells, the positive control that we have used is trichloroethylene. Baseline viability in the absence of trichloroethyelene is between 75 and 95%. This is based on approximately 26 experiments, or 20 different bone marrow cell preparations. The cell number at the end of the exposure of the control cultures is 1.5 to 4.0×10^6 cells/ml. There is more variability in the bone marrow cells than in the spleen cells. Trichloroethylene at 10^{-2}M should produce about a 50% reduction in the trypan blue exclusion, and 600 μg/10^6 bone marrow cells should produce a 50% reduction in the cell number.

Baseline and ED50 values of positive controls for the DNA synthesis in bone marrow cells were also determined. The baseline cpm's after 1 hour incubation with IUdR ranged between 8,000 and 40,000. Again, this variability between experiments is most likely a function of the status of the mice from which the bone marrow cells are taken. Cytosine arabinoside represents an excellent positive control in this system, with an ED50 of 8 μg/10^6 cells. Cyclophosphamide will inhibit DNA synthesis in the bone marrow cells, with an ED50 of approximately 110 μg/10^6 cells in the presence of an induced S9 mix. Again, the effect of cyclophosphamide on DNA synthesis is not as effective as the anti-metabolite cytosine arabinoside.

Baseline and ED50 values of the positive controls for bone marrow stem cells have been calculated. We have now run 38 assays on the bone marrow stem cells, and the range of stem cells/5×10^5 bone marrow cells is between 110 and 220. The ED50 for cyclophosphamide in the presence of the S9 mix for the stem cells is 5 μg/10^6 bone marrow cells. For cytosine arabinoside, the ED50 is 15 μg/10^6 bone marrow cells. These two agents represent excellent positive controls for this particular assay.

GENERAL STATISTICAL APPROACH

For the chemical effects, analysis of variance was used to investigate the effects of various factors. The model employed was:

$$X_{ijklm} = \mu + D_i + F_{j(i)} + T_k + I_e + DT_{ik} + DT_{ie}\ TI_{ie}\ DTI_{ike} + E_{ijklm},$$

where D_i + effect of chemical concentration $F_{j(i)}$ = nested effects of the tubes, T_k = IUdR time effect, I_e = incubation time effect, and the other terms are the interaction effects. Note that the interaction effects with tubes are assumed zero. The model assumed for the analysis was:

$$X_{ijklm} = \mu + D_i + F_{j(i)} + S_{k(i,j)} + T_e + DT_{ie} + E_{ijklm},$$

where D_i = the effect of chemical concentration $F_{j(i)}$ = nested effects of tubes, $S_{k(i,j)}$ = nested sample effect, T_e = IUdR time effect, and DT = dose and time interaction effect. The interaction effects with tubes and samples are assumed zero.

The ED50 with confidence limits were calculated by the model described by Box and Hunter (1954). The model from which the model was derived from is

$$y = B_0 + B_1 \times LOG_{10} (dose + 1)$$

PART II - IN VIVO STUDIES

Sections A through C of Part II (General Approach and Methods, General Toxicology of Trichloroethylene, and Effects of Trichloroethylene on the Immune System) represent manuscripts that have been published in the peer reviewed literature. Section A appeared in Drug and Chemical Toxicology, and Sections B and C appeared in Toxicology and Applied Pharmacology.

IMMUNOTOXICOLOGICAL INVESTIGATIONS IN THE MOUSE

A. GENERAL APPROACH AND METHODS

ABSTRACT

The adverse effects of chemicals on the lymphoreticular system have generated considerable toxicological interest. In this series of papers, the effects of selected environmentally relevant compounds are reported. This first paper describes the methods and general approach used in judging a chemical's potential risk to the immune system. Risk evaluation was approached utilizing acute, 14- and 90-day studies. Both sexes of the CD-1 random-bred mouse were employed. The immune system was evaluated against a background of more standard toxicological parameters, which included fluid consumption, body and organ weights, hematology, serum and liver chemistries, hepatic microsomal enzyme activities and blood coagulation. Bone marrow status was evaluated by assessing DNA synthesis. Humoral immunity was evaluated by determining the number of IgM spleen antibody-forming cells (AFC) to sheep erythrocytes (sRBC), the serum antibody level to sRBC, and spleen lymphocyte response to the B cell mitogen, lipopolysaccharide (LPS). The status of cell-mediated immunity was assessed by quantitating the delayed type hypersensitivity (DTH) response to sRBC, proliferation of the popliteal lymph node, and the spleen cell response to the T lymphocyte mitogen, Concanavalin A (Con A). Macrophage function was evaluated by measurement of the vascular clearance rate and distribution of radiolabeled sRBC in the liver, spleen, lungs, and thymus, and recruitability, adherence, chemotaxis, and phagocytic activity of peritoneal exudate cells (PEC). Historical control data from six 14- and 90-day studies conducted over a one year period are given. The data resulting from these types of studies can provide a basis for the initial evaluation of a chemical's adverse effect on the immune system.

INTRODUCTION

The adverse effects of chemicals on the organs, tissues, and cells of the lymphoreticular system have received considerable attention from both toxicologists and immunologists. This interest is well founded since the immune system has been shown to be the target organ of certain chemically and physically diverse compounds (Koller, 1979; Thomas and Hinsdall, 1979; Vos et

al., 1979). This system, like all other systems in the body, is complex, with several types of cells working both independently and in concert, to carry out a role in homeostasis. Immunotoxicology is a subject gaining increased activity and awareness because the physiology and biochemistry of the immune system are now being systematically dissected; as a result, the basic processes involved in host defense mechanisms are better understood. A second reason for the interest in immunotoxicology is that it represents a system in which the cells from exposed animals can be readily removed and their function(s) examined in vitro. This is in keeping with the direction that toxicology is taking, i.e., complementing morphological changes with functional alterations.

This paper describes the approach we have taken in evaluating the immunotoxicity of chemicals in experimental random-bred mice. The effects of a given chemical on the immune system were investigated against a background of standard toxicological procedures. In this way, the specificity of the immune system as a target could be more clearly defined. Historical control data are provided for each of the assays, with the data presented as the mean ± SD. In addition, an analysis of variance was calculated on the combined experiments to determine if there were significant differences among the control values from experiment to experiment.

EXPERIMENTAL APPROACH

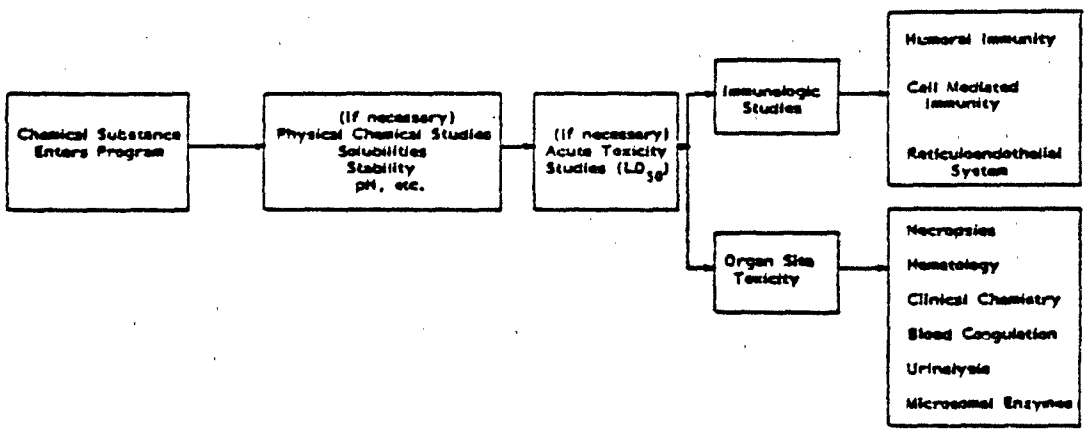
Figure 26 outlines the experimental design which is now being used to evaluate chemicals for their target organ toxicity.

After the compound was selected for study, unknown physical and chemical properties of the chemical were determined and necessary studies were performed to assure appropriate integration into the in vivo systems. These data included: confirmation of identity, solubility properties, pH characteristics, stability at concentrations in the test system, and purity.

Acute toxicity studies were performed on eight week old male and female mice using the route of administration which most closely approximates human exposure. Routinely, eight mice were used per group and at least six dose levels were employed. In many of these studies, we were concerned with environmental chemicals that are introduced orally. The mice were exposed via an 18 gauge stainless steel stomach tube after 18 hr of fasting. The mice were observed hourly for the first eight hours for behavioral changes and morbidity, then twice daily thereafter for 14 days. The animals still alive

FIGURE 26

FLOW CHART FOR IMMUNOTOXICOLOGICAL STUDIES



at the end of the experimental period were sacrificed, and all mice were necropsied and examined for gross pathology. Calculations of the LD_{50} and slopes of the dose-response curves with 95% confidence limits were performed by the Probit Procedure of Goodnight (1979). These data provided the basis for selecting the dose levels employed in the 14- and 90-day studies, and began to indicate the possible target organ(s).

Following the acute studies, a 14-day range finding study was performed. If no sex differences were seen in the acute toxicity, only male mice were used. Again, the relevant route of exposure was employed. The chemical was administered daily for 14 days via stomach tube at two dose levels. These levels were usually 1/10 and 1/100 the LD_{50}. Body weights were determined and recorded prior to the initiation of exposure, and again one and two weeks after exposure.

Table 20 lists the variables monitored after 14 days of exposure. Three sets of mice were used to obtain this toxicological information. In one set of mice, humoral immunity was assessed by enumerating the number of spleen IgM AFC to sRBC, both as a function of AFC per spleen and per 10^6 spleen cells. In the same mice, serum obtained from blood collected by cardiac puncture was used for serum chemistry studies. These included glutamic-pyruvic transaminase, lactate dehydrogenase and blood urea nitrogen. A second set of mice was used for general toxicological assessment. Cardiac blood was collected into 3.2% sodium citrate (1:10 citrate to blood) for hematology and coagulation studies. Hematological studies included leukocyte counts, hematocrit, and hemoglobin levels. If a change was seen in leukocyte counts, a differential analysis was performed. A complete necropsy was performed on this set of mice and the following organs were removed, trimmed, and weighed: brain, liver, spleen, lungs, thymus, kidneys, and testes. The organ weight data were expressed as both organ to body weight ratio and as organ to brain weight ratio. Cell-mediated immunity was assessed in the third set of mice by measurement of the DTH response to sRBC.

Tables 21-25 show the control values for CD-1 mice in the 14-day studies. These values were derived from six 14-day studies performed over a one-year period. The mice were five weeks of age when they arrived at our facility. They were quarantined for seven days and then placed in the study for the two-week period. The 14-day study provided baseline data for the design of the 90-day study. Except for mutagenic, carcinogenic, teratogenic, and reproduct-

ive effects, the 90-day study should successfully describe the target organ toxicity.

TABLE 20

Parameters Measured in a 14-Day Study

Standard Toxicology

1. Body Weights
2. Necropsy - Gross Pathology
3. Organ Weights
4. Hematology
5. Serum Chemistries
6. Blood Coagulation

Immunotoxicology

1. Humoral immunity - spleen AFC response to sRBC
2. Cell-mediated immunity - DTH response to sRBC

Doses were based on the acute toxicity study and were usually 1/10 and 1/100 of LD50.

TABLE 21

Control Values for Body Weights (Grams) of Two Month Old Male CD-1 Mice

Final Weight	Initial Weight	Change in Weight
30.0 ± 2.8	24.2 ± 2.3	5.8 ± 2.3

Values represent the mean ± SD of 334 control male CD-1 mice derived from six 14-day experiments. All weight means were significantly different ($p < 0.05$) among the six experiments.

TABLE 22

Control Organ Weight Values of Two Month Old Male CD-1 Mice

Organ	Weight (mg)	% Body Weight	Organ/Brain
Brain	440 ± 39	1.47 ± 0.15*	-
Liver	1862 ± 294*	6.17 ± 0.73	4.26 ± 0.74
Spleen	170 ± 53	0.56 ± 0.17	0.39 ± 0.12
Lungs	214 ± 35	0.71 ± 0.11	0.48 ± 0.07
Thymus	79 ± 19	0.26 ± 0.06	0.18 ± 0.04*
Kidney	522 ± 84	1.73 ± 0.20	1.19 ± 0.15
Testes	211 ± 34	0.70 ± 0.11	0.48 ± 0.07*

Values represent the mean ± SD of 68-70 control male CD-1 mice derived from six 14-day experiments. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

TABLE 23

Control Values for Selected Hematological, Serum Chemistry and Blood Coagulation Parameters in Two Month Old Male CD-1 Mice

Parameter	Values
Hemoglobin (g%)	12.7 ± 2.1
Hematocrit (%)	41.2 ± 2.5
Leukocytes ($10^3/mm^3$)	7.44 ± 2.76
Lactate Dehydrogenase (IU/L)	926 ± 195
Glutamic-Pyruvic Transaminase (IU/L)	56.4 ± 21.6
Blood Urea Nitrogen (mg%)	27.2 ± 4.8
Prothrombin Time (sec)	8.4 ± 0.8
Fibrinogen (mg%)	298 ± 41

Values represent the mean ± SD derived from 58 control mice used in six 14-day studies. All values were significantly different ($p < 0.05$) among the six experiments.

TABLE 24
Control Values for Humoral Immune Assessment in Two Month Old Male CD-1 Mice

Parameter	Values
Spleen Weight (mg)	204 ± 47
Spleen Cell Number ($\times 10^{-8}$)	1.65 ± 0.48
IgM AFC/Spleen ($\times 10^{-5}$)	4.86 ± 3.18
IgM AFC/10^6 Spleen Cells	2904 ± 1667

Values represent the mean ± SD derived from 68 control mice in six 14-day studies. The antibody forming cells were enumerated on the peak day of response (day 4). All parameters were significantly different ($p < 0.05$) among the six experiments.

TABLE 25
Control Values for the DTH Response to sRBC in Two Month Old Male CD-1 Mice

Parameter	Value
Stimulation Index	3.73 ± 1.60

The value represents the mean ± SD derived from 70 control mice used in six 14-day studies. A value of 1.97 has been subtracted from each animal value to correct for non-specific swelling as described in Methods. There was no significant difference ($p < 0.05$) among the six experiments.

Table 26 lists the variables that were measured during, and at the conclusion of, the 90-day study. Six sets of mice were used to evaluate these variables. The first set was used for necropsy (gross pathology), organ weights, microsomal mixed functional oxidase parameters, liver glutathione levels, hematology, coagulation, hemagglutination titer to sRBC and bone marrow DNA synthesis. The second set of mice was used to determine spleen AFC response to sRBC, spleen cell response to mitogens, and serum chemistries. Assessment of cell-mediated immunity, as measured by the DTH response to sRBC, was accomplished with the third set of mice, while the fourth set of mice was used to determine popliteal lymph node proliferation. The fifth set of mice was used to measure the functional activity of the reticuloendothelial system, while the sixth set of mice was used to study the number and functional status of PEC. Tables 27-37 show the control values for CD-1 mice in the 90-day studies.

TABLE 26
Parameters Measured in a 90-Day Study

A. Standard Toxicology

1. Body weights - twice weekly
2. Fluid consumption
3. Necropsy - gross pathology
4. Organ weights - brain, liver, spleen, lungs, thymus, kidneys, and testes
5. Hematology - hematocrit, erythrocytes, leukocytes, differential, platelets, and hemoglobin
6. Coagulation - prothrombin time, activated partial thromboplastin time, and fibrinogen
7. Serum chemistries - lactate dehydrogenase, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, alkaline phosphatase, blood urea nitrogen, total protein, glucose, cholesterol, creatinine, phosphorus, calcium, sodium, chloride, and potassium
8. Liver chemistries - glutathione levels

B. Hepatic Microsomal Mixed Functional Oxidase Parameters

1. Liver weight
2. Microsomal protein
3. P450 content
4. Cytochrome b_5
5. Aminopyrine-N-demethylase
6. Aniline hydroxylase

C. Cell Mediated Immunity

1. DTH response to sRBC
2. Popliteal lymph node proliferation to sRBC
3. Spleen cell response to Con A

D. Humoral Immunity

1. Spleen AFC response to sRBC
2. Serum antibody response to sRBC
3. Spleen cell response to LPS

E. Functional Activity of the Reticuloendothelial System

1. Vascular clearance rate of sRBC
2. Organ uptake of sRBC
3. Chemotaxis, adherence, and phagocytic activity of PEC

F. Bone Marrow

1. DNA synthesis

METHODS

Experimental Animals

Although the mouse is currently being used for some toxicological studies, the random bred rat has been the experimental animal most widely used and considered to be the most appropriate for toxicological evaluation of chemicals and drugs. Unfortunately, the physiology of the rat's immune system has not been studied or defined to the same degree as that of the mouse and man. The mouse has been the immunologist's animal of choice because of the availability of inbred strains, which has allowed for the development of specific immune sera and for decreased animal-to-animal variation. In addition, due to the homogeneity of the lymphoid cells derived from genetically similar inbred mice, functional tests can be performed on cell samples pooled from several animals.

As a compromise, the program we have developed employs the random bred CD-1 mouse. Of the three commercial sources of mice we evaluated, only one (Charles River Breeding Laboratories, Wilmington, MA) supplied mice of consistent quality and temperament. This mouse has been used to assess the effects of chemicals on the immune systm, and with the increased availability of microassays, has become attractive for the more routine toxicological tests.

Upon arrival, all animals were housed four per cage in plastic shoe box cages containing sawdust bedding (PWI Hardwood Sawdust, Lowville, NY). After the quarantine period (7 days), animals were randomized and were individually earpunched. Acute toxicity studies were performed on animals eight weeks of age. The 14-day studies were begun on animals six weeks of age, while four week old animals were used in 90-day studies. Mice exposed by gavage were maintained on Purina Lab Chow and water ad libitum. Those animals exposed to the chemicals in drinking water were maintained on Purina Lab Chow and chemical solutions ad libitum. Control values for body weights of the mice used in the 90-day studies are presented in Table 27. Animal room temperature was maintained between 21 and 24°C and relative humidity between 40-60%. The light-dark cycle was maintained on 12-hr intervals.

If the chemical was water soluble it was placed directly in deionized water. For the chemicals which were water insoluble, a polyethoxylated vegatable oil, emulphor (GAF 620, GAF Corp., New York, NY), was used as the

TABLE 27

Control Values for Body Weight (Grams) of Male and Female CD-1 Mice Used in 90-Day Studies

	Final Weight	Initial Weight	Change in Weight
Males	39.3 ± 4.6	22.2 ± 3.6	17.3 ± 5.7
Females	30.6 ± 3.8	19.5 ± 2.8	11.1 ± 4.5

Values represent the mean ± SD of 288 male and 288 female control mice derived from six 90-day studies. All weights were significantly different ($p < 0.05$) among the six experiments.

vehicle. Ten percent emulphor in deionized water was used to dissolve chemicals used in gavage studies, while a 1% emulphor solution was used for drinking water studies, if necessary. Solutions for gavaging were prepared fresh daily, while solutions for the drinking water were prepared twice weekly. All solutions were maintained in dark bottles to alleviate possible photodecomposition. Gavaging was done with an 18 gauge stainless steel feeding tube between the hours of 10:00 and 11:00 am Solutions for drinking water studies were placed in 250-500 ml amber colored bottles capped with cork stoppers containing stainless steel sipper tube inserts. All drinking water solutions were analyzed by GLC with head space analysis to determine the stability of the compound over a three- to four-day period at room temperature. Fluid consumption was calculated by weighing the water bottles at the time solutions were initially placed in them, then weighing the bottles again before solutions were changed to determine the amount consumed over the three- or four-day period.

Blood and Tissue Collection, Hematology, and Coagulation Studies

To assure that the maximum amount of blood was obtained and that its quality was applicable for special coagulation tests, the mice were anesthetized with chloroform. Several anesthetic agents have been tried, and the two that did not perturb any of the parameters examined, including the hepatic microsomal mixed functional oxidase system, were chloroform and sodium brevital. Sodium pentobarbital and hexobarbital perturbed liver microsomal enzymes and ether affected the quality and amount of blood drawn from the animals

(data not shown). The blood was collected by cardiac puncture into a 3 ml plastic syringe fitted with a 23 gauge needle. For the hematology and blood coagulation studies, the blood was collected into 3.2% sodium citrate (1:10 citrate to blood). The samples were collected without air bubbles within 30 sec of the puncture. One to two ml of blood were routinely collected by this method. The blood was maintained at 4°C and leukocytes, erythrocytes, and platelets were enumerated on a Coulter counter, Model ZBI. Platelet counts were determined on platelet-rich plasma by a modification of the methods of Bull et al. (1965). Leukocyte differentials were evaluated using the standard Wright's-Giemsa staining procedure. Hematocrits were performed on a micro-hematocrit centrifuge and hemoglobins determined by the cyanomethemoglobin method. The plasma was separated from the cellular elements by 800 xg centrifugation at 4°C. The plasma was used to evaluate the status of the coagulation system. Prothrombin times were performed to assess extrinsic activity and activated partial thromboplastin times (APTT) were performed to assess intrinsic activity. These assays were done on a BBL Fibrometer using General Diagnostics reagents (Baltimore, MD). Fibrinogen levels were determined by the kinetic method using Dade Diagnostics reagents (Miami, FL). Control historical hematologic and coagulation data from six 90-day studies are shown in Table 28. The only parameter showing a sex difference was the fibrinogen level, where the females had a 25% lower level than the males.

Immediately after the blood sample was drawn, the liver was examined, removed, trimmed, weighed, and if needed, a section was removed for histopathological examination. The remaining liver tissue was homogenized and a microsomal fraction was prepared for analysis. One femur was removed and the bone marrow collected for analysis. A complete necropsy was then performed and the following organs were removed, trimmed, and weighed: brain, liver, spleen, lungs, thymus, kidneys, and testes. Samples of these tissues were fixed in 10% buffered formalin along with the heart, mesenteric lymph nodes, striated muscle, stomach, ileum, jejunum, colon, bladder, ovaries, and adrenals. The most notable sex differences in organs weights were in the brain and kidney (Table 29). The brain was 28% larger in the female when calculated on a percent body weight basis. The kidney was 17% and 35% larger in the male when calculated on a percent of body weight and organ to brain ratio, respectively.

TABLE 28

Control Hematology Values for Male and Female CD-1 Mice in 90-Day Studies

Parameter	Male	Female
Hemoglobin (g%)	12.5 ± 2.0	12.3 ± 2.0
Hematocrit (%)	41.3 ± 3.7	41.4 ± 2.0*
Erythrocytes (10^6/mm^3)	8.19 ± 1.59	8.51 ± 2.05
Leukocytes (10^3/mm^3)	6.42 ± 2.79	6.37 ± 2.94
Platelets (10^5/mm^3)	3.78 ± 0.90	3.60 ± 0.81
Prothrombin time (sec)	9.6 ± 0.7	9.7 ± 0.7
APTT	31.1 ± 5.5*	33.1 ± 5.8*
Fibrinogen (mg%)	282 ± 50	211 ± 29
Differential:		
Lymphocytes (%)	67.2 ± 15.8	78.7 ± 9.0
Polymorphonuclears (%)	30.0 ± 15.4	16.1 ± 7.9
Monocytes (%)	3.8 ± 3.3	3.6 ± 2.8
Eosinophils (%)	1.0 ± 1.3*	1.6 ± 1.2*

Hematology values represent the mean ± SD of 131 male and 137 female control CD-1 mice derived from six 90-day studies. Differential values represent the mean ± SD of 88-92 male and female CD-1 mice derived from four subchronic 90-day studies. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

Preparation of Microsomes

Immediately following sacrifice, livers were removed, weighed, thoroughly rinsed, and homogenized in 9 volumes of ice cold 0.15M KCl - 0.01M potassium phosphate buffer (pH 7.4) using a teflon glass homogenizer, and centrifuged at 9000 xg for 20 min. An aliquot of the supernatant fluid was removed, carefully avoiding the top lipid layer, and recentrifuged at 105,000 xg for 60 min. The resulting pellet was rinsed and resuspended in 0.05M potassium phosphate buffer to obtain a concentration of approximately 2 mg protein per mg of buffer. The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (1951), using human serum albumin as the standard.

TABLE 29
Control Organ Weight Values for CD-1 Mice
Used in 90-Day Studies

Organ	Weight (mg)	% Body Weight	Organ/Brain
		MALES	
Brain	499 ± 39	1.27 ± 0.14	-
Liver	2057 ± 349	5.17 ± 0.53	4.13 ± 0.70
Spleen	167 ± 69	0.42 ± 0.16	0.34 ± 0.14
Lungs	234 ± 36	0.59 ± 0.08*	0.47 ± 0.07
Thymus	45 ± 14	0.11 ± 0.03	0.09 ± 0.03
Kidney	650 ± 102	1.64 ± 0.18*	1.30 ± 0.20
Testes	260 ± 41	0.66 ± 0.10*	0.52 ± 0.08*
		FEMALES	
3rain	497 ± 41	1.63 ± 0.22	-
Liver	1526 ± 242	4.97 ± 0.68	3.10 ± 0.59
Spleen	155 ± 45	0.50 ± 0.13	0.32 ± 0.10
Lungs	216 ± 40	0.71 ± 0.13	0.44 ± 0.09
Thymus	54 ± 16	0.17 ± 0.05	0.11 ± 0.03
Kidney	418 ± 54	1.36 ± 0.16	0.85 ± 0.13

Values represent the mean ± SD of 132 male and 138 female control CD-1 mice derived from six 90-day studies. All values were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

Drug Metabolism Assays

Substrates were selected as representative of Type I (aminopyrine) and Type II (aniline) cytochrome P450-dependent mixed function oxidase. Historical control data for drug metabolism assays are shown in Table 30.

Aminopyrine N-demethylase: The methods of Cochin and Axelrod (1959) were used to measure the in vitro metabolism of aminopyrine. A 25 ml Ehrlenmeyer flask containing 0.5 ml cofactors and buffer (7.5 µmol $MgCl_2 \cdot 6H_2O$, 17 µmol glucose-6-phosphate, 2 µmol NADP, and 1 unit glucose-6-phosphate dehydrogenase in 0.5M potassium phosphate buffer, pH 7.4), 0.5 ml aminopyrine (10 µmol), and 1.0 ml of microsomal suspension (2 mg protein/ml) was prepared. Blanks were prepared similarly, except that aminopyrine was omitted from the flasks. The

TABLE 30

Control Values for Hepatic Microsomal Mixed Functional Oxidase Parameters of Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Unit	Male	Female
Microsomal Protein	mg/g liver	23.4 ± 3.2	20.4 ± 2.3
Cytochrome P450	nmol/mg protein	1.16 ± 0.259	0.98 ± 0.184
Cytochrome b5	nmol/mg protein	0.41 ± 0.050	0.52 ± 0.079
Aminopyrine N-Demethylase	nmol/mg protein/min	10.6 ± 1.7	13.1 ± 2.1
Aniline Hydroxylase	nmol/mg prot/min	1.60 ± 0.48	1.72 ± 0.37

Values represent the mean ± SD of 44 male and 48 female mice from six 90-day studies. The means of the parameters were significantly different ($p < 0.05$) among the experiments.

flasks were shaken for 10 min at 37°C at 120 oscillations per min in a gyratory shaker bath. At the end of the incubation period, the reaction was terminated by the addition of 0.6N perchloric acid. The contents were transferred to centrifuge tubes and centrifuged at 2000 xg for 15 min. One ml of the supernatant fluid was added to 1 ml of double strength Nash reagent (300 g ammonium acetate and 2 ml acetyl acetone per liter H_2O), and the color allowed to develop for 30 min in a 60°C water bath. Samples were cooled immediately and the absorbance at 415 nm was recorded.

Aniline Hydroxylation: The 4-hydroxylation of aniline was determined using a comparable procedure (Imai et al., 1966), except that 16 µmol of aniline HCl was incubated in the presence of cofactor and microsomes. The reaction was terminated by addition of 1.0 ml of 20% trichloroacetic acid. One ml of the protein-free supernatant fluid was added to 0.5 ml 10% sodium carbonate, mixed, and added to 1.0 ml of phenol reagent (2% phenol in 0.2N NaOH). The color was allowed to develop at 37°C for 30 min and the absorbance at 630 nm recorded.

Spectral Studies

The methods of Omura and Sato (1964) were used to measure the content of microsomal cytochromes P450 and b_5. Historical control data are shown in Table 30.

Cytochrome P450: Microsomes were diluted to 1 mg protein per ml in 0.1M potassium phosphate buffer. Carbon monoxide was gently bubbled through the sample cuvette for 30 sec and a few crystals of sodium dithionite were added to each cuvette. The CO-difference spectrum of the reduced microsomes was recorded from 500-400 nm and cytochrome P450 content was calculated from the absorbance difference (OD 450 nm minus OD 490 nm) using 91 mM^{-1} cm^{-1} as the mM extinction coefficient.

Cytochrome b_5: Microsomes were diluted to 1 mg protein per ml buffer and divided equally into two cuvettes. The sample cuvette was reduced by adding 4.4 µmol NADH in 0.01 ml H_2O. The reduced vs oxidized spectrum was recorded and cytochrome b_5 content calculated from the absorbance difference (OD 423 nm minus OD 409 nm), using 185 mM^{-1} cm^{-1} as the mM extinction coefficient.

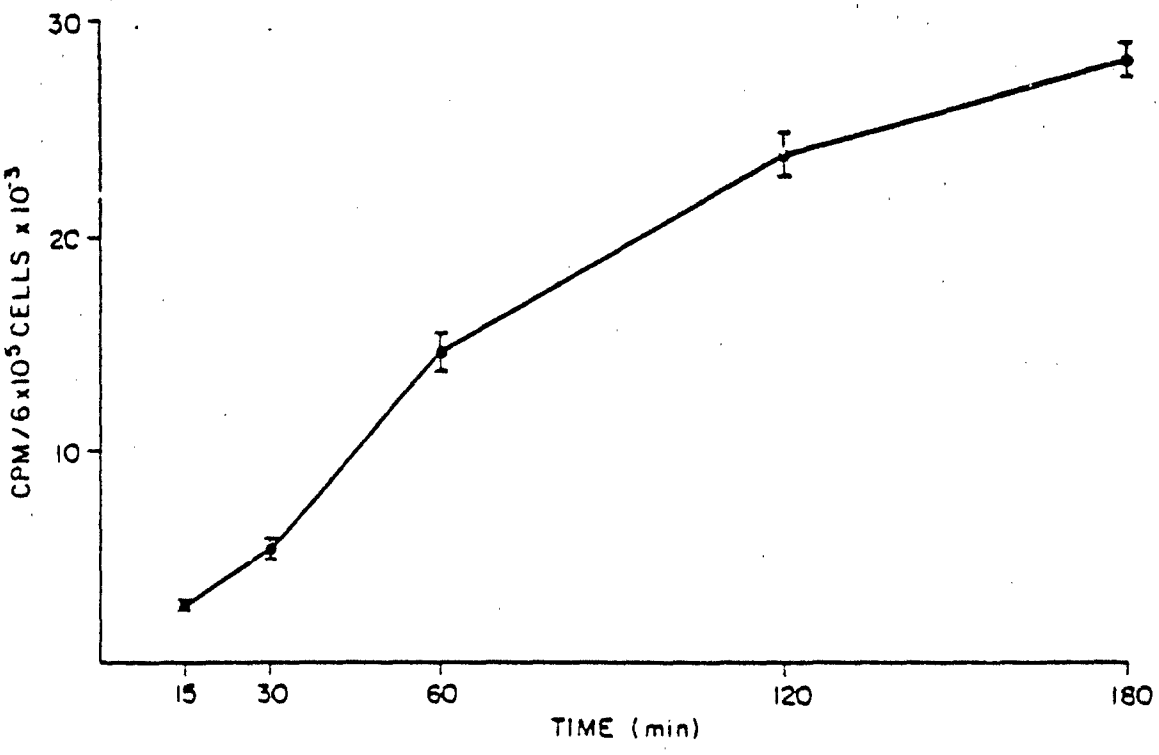
Bone Marrow

The status of the bone marrow was evaluated by determining bone marrow cell DNA synthesis. In addition, bone marrow smears were prepared for evaluation if marked changes in the parameter occurred. Bone marrow cells were added to each well of a 96-well microtiter dish at 6 x 10^5 cells/200 µl. Twenty µl of a solution containing 0.1 µCi of ^{125}I-Iododeoxyuridine (^{125}I-IUdR; New England Nuclear) in 2 x 10^{-5}M Fluorodeoxyuridine (FUdR; Sigma Chemical Co.) was added to each well. At 60 and 120 min, triplicate wells were harvested and the cells were collected onto filter disks with the aid of a Titertek cell harvester. Filter disks containing the ^{125}I-IUdR incorporated into DNA were radioassayed in a Beckman 300 gamma counter. Figure 27 shows a typical incorporation curve for ^{125}I-IUdR into bone marrow cells. As can be seen, there is a linear incorporation of ^{125}I-IUdR over a 3 hr incubation period. Appropriate studies were performed to assure that ^{125}I-IUdR was incorporated into DNA. Table 31 shows control historical data on bone marrow DNA synthesis.

Serum and Liver Chemistries

The serum enzymes, glutamic-pyruvic transaminase, glutamic-oxaloacetic transaminase, lactate dehydrogenase, and alkaline phosphatase were measured kinetically on the ABA 100 Bichromatic analyzer (Abbott Laboratories, Dallas,

FIGURE 27



The time course of incorporation of ^{125}I-IUdR into bone marrow cells over a 180 min. period. See Methods for procedure.

TABLE 31

Control Values for Bone Marrow DNA Synthesis from Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Male	Female
DNA Synthesis (60 min)	16082 ± 4411	17246 ± 4319
DNA Synthesis (120 min)	29563 ± 6655	30224 ± 8823

Values represent the mean ± SD of 60 male and female mice from six 90-day studies. There were no significant differences ($p < 0.05$) among the six experiments.

TX). These enzymes had been optimized for the mouse as to pH and substrate concentration. A-GENT reagent kits purchased from Abbott Laboratory Diagnostic Division (South Pasadena, CA) were used to determine blood urea nitrogen, total protein, glucose, cholesterol, and calcium, while Pierce reagent kits (Pierce Chemical Co., Rochford, IL) were used to measure phosphorus and creatinine. These chemistries were measured by endpoint analysis on the above-named instrument. Sodium and potassium ions were measured on a flame photometer (Instrumentation Laboratory 443), and chloride ions were measured on a chloridometer (Buchler Instruments). The methods of Jollow et al. (1974) were used to measure liver glutathione content. A mixture of 0.5 ml of liver homogenate (described previously) and 0.5 ml of 4% sulfosalicylic acid was centrifuged at 2000 xg for 15 min. Two hundred µl of protein-free supernatant fluid were added to 2.0 ml of 0.4 mM Ellman's Reagent (5,5'-dithiobis-2-nitrobenzoic acid) in 0.1M potassium phosphate buffer, pH 8.0. Ten min after mixing, the absorbance at 412 nm was recorded and compared to the absorbance of glutathione standards. Baseline historical control data for the serum and liver chemistries from the 90-day studies are shown in Table 32.

Cell-Mediated Immunity

The functional status of both the afferent and efferent arms of cellular immunity was evaluated by measuring a DTH response to sRBC. Sheep erythrocyte sensitization was done on Day 0 in the left hind footpad (LFP) with 1×10^8 sRBC in a volume of 20 µl. Four days following sensitization, the mice were

TABLE 32

Control Serum and Liver Chemistry Values in Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Unit	Male	Female
LDH	IU/L	664 ± 233	606 ± 193
SGPT	IU/L	44.6 ± 24.4	36.5 ± 18.6
SGOT	IU/L	84.4 ± 38.9	92.0 ± 28.2
SAP	IU/L	44.1 ± 22.6	62.4 ± 29.7
Glutathione	µmol/g liver	8.97 ± 1.79	7.85 ± 1.66
BUN	mg%	27.7 ± 6.9	22.8 ± 5.2
Protein	g	7.13 ± 1.21	7.56 ± 1.08
Glucose	mg%	160 ± 32	137 ± 24
Cholesterol	mg%	167 ± 98	120 ± 77
Creatinine	mg%	0.42 ± 0.19	0.50 ± 0.23
Phosphorus	mEq/L	8.40 ± 2.01	8.05 ± 2.28
Calcium	mEq/L	12.0 ± 2.1	11.7 ± 1.6
Sodium	mEq/L	155 ± 10	156 ± 11
Chloride	mEq/L	108 ± 12	109 ± 14
Potassium	mEq/L	7.30 ± 0.94	6.90 ± 0.95

Values represent the mean ± SD derived from 70-143 control CD-1 mice used in six 90-day studies. LDH=lactate dehydroge nase, SGPT=serum glutamic-pyruvic transaminase, SGOT=serum glutamic-oxaloacetic transaminase, SAP=serum alkaline phospha tase, BUN=blood urea nitrogen. The means of each parameter were significantly different ($p < 0.05$) among the six experiments.

challenged in the same footpad with 4×10^8 sRBC in a volume of 40 µl. Seventeen hr following the challenge, mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (^{125}I-HSA; 80,000 cpm/0.1 ml; Mallinckrodt). Two hr later, the mice were sacrificed by cervical dislocation and both hind feet were removed at the ankle joint and radioassayed in a gamma counter. It has been shown that ^{125}I-HSA will extravasate into the edematous area produced by a DTH response (Paranjpe and Boone, 1972). The right hind footpad (RFP) served as a control for background infiltration of ^{125}I-HSA. A group of mice which were unsensitized but challenged as above acted as unsensitized

controls to determine non-specific swelling. Results are expressed as a stimulation index (SI), which was calculated are follows:

$$SI = \frac{\text{LFP sensitized}}{\text{RFP sensitized}} - \text{mean } \frac{\text{LFP unsensitized.}}{\text{RFP unsensitized}}$$

Evaluation of cell-mediated immunity using the footpad assay requires both the afferent arm (antigen recognition and processing, blastogenesis) and the efferent arm (lymphokine production, increased vascular permeability) to function adequately. If an experimental chemical decreases the extravasation of ^{125}I-HSA, it may be evaluated as immunosuppressive in the cell-mediated response. However, this may not necessarily be true if the compound has anti-inflammatory properties. To determine if a chemical affects the afferent arm of the cellular response, proliferation of the popliteal lymph node cells to sRBC was measured. Additional groups of animals were used for this assay. They were subjected to the same procedure as were the DTH animals, except that 1 1/2 hr after challenge they received 20 µg/kg FUdR intraperitoneally, and 2 hr after challenge they received 1 µCi of ^{125}I-IUdR intravenously. ^{125}I-HSA was not administered to these animals. These mice were sacrificed 24 hr after challenge and both popliteal lymph nodes were removed and counted in a gamma counter. Non-specific proliferation was corrected for and an SI was calculated as described for the footpad assay. Historical control data for both footpad swelling and popliteal lymph node proliferation are shown in Table 33.

Lymphocyte Responsiveness

The mitogens employed were Con A, a T cell mitogen in the mouse, and LPS, a B cell mitogen. A 0.05 ml volume of RPMI 1640 medium containing either 1, 5, or 10 µg of Con A or 1, 5, or 20 µg of LPS was placed in flat-bottom microtiter wells and frozen at -70°C. When needed, plates were removed from the freezer and thawed at room temperature. No differences were observed between mitogen plates frozen and those freshly prepared. To detect a maximal mitogen response and to detect any shifts in the dose response curve, three concentrations of mitogen were routinely used.

Spleens were aseptically removed from mice and single cell suspensions prepared by pushing spleens through sterile 60-mesh wire screens into RPMI 1640 media supplemented with 5% heat-inactivated fetal calf serum (HIFCS), 2 mM L-glutamine, 100 µg/ml penicillin and 1 µg/ml streptomycin. The cell con-

TABLE 33

Control Values for Cell-Mediated Immune Response as Measured by DTH and Popliteal Lymph Node Proliferation to sRBC in Male and Female CD-1 Mice Used in 90-Day Studies

Stimulation Index	Male	Female
Footpad Swelling	4.02 ± 2.05	4.61 ± 2.15
Popliteal Lymph Node Proliferation	18.0 ± 12.1	12.0 ± 6.7*

Footpad swelling values represent the mean ± SD of 103 male and 104 female mice derived from six 90-day studies. A value of 1.95 for males and 2.07 for females has been subtracted from each animal to correct for non-specific swelling as described in Methods. Popliteal lymph node data were obtained from three 90-day studies using 29 males and 32 females. To correct for non-specific lymph node proliferation, a value of 1.1 for males and 1.3 for females was subtracted from each animal. The means of each parameter were significantly different ($p < 0.05$) among the experiments, except where indicated by an asterisk.

centration was adjusted to 5×10^6 cells/ml in RPMI medium supplemented with 10% HIFCS, L-glutamine, and pencillin-streptomycin. One tenth ml of the cell suspension was placed in thawed microtiter wells containing mitogen; additionally, wells were set up without mitogen to act as background controls.

The plates were then placed in a 10% CO_2, 37°C humidified incubator. After 48 hr, 0.05 ml of a radiolabeled solution was added to each well. This solution contained ^{125}I-IUdR (4 μCi/ml) and FUdR (4×10^{-6}M). The plates were reincubated for 18-20 hr, at which time the cells were collected on filter disks using a Titertek cell harvester. The filter disks were then counted in a gamma counter. There are two ingredients which were essential for this assay to work: heat-inactivated fetal calf serum and FUdR.

Historical control data for lymphocyte responsiveness to Con A and LPS are shown in Table 34.

Humoral Immunity

Hemolytic Plaque Assay: The method used in our laboratory to detect AFC was the Cunningham modification of the Jerne plaque assay (Cunningham and Szenberg, 1968). IgM AFC were enumerated 4 and 5 days following intraperitoneal immunization with 4×10^8 sRBC. Data are presented as AFC/10^6

TABLE 34
Control Values for Spleen Lymphocyte Responsiveness to Con A and LPS in Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Male (cpm/5 x 10^5 cells)	Female (cpm/5 x 10^5 cells)
No mitogen	7778 ± 4959	10179 ± 6601
Con A (1 µg)	97654 ± 58212	118697 ± 53177
Con A (5 µg)	165116 ± 68393	195746 ± 62703
Con A (10 µg)	55920 ± 74316	51333 ± 74816
LPS (1 µg)	51064 ± 33406	83905 ± 42547
LPS (5 µg)	53138 ± 33170	90994 ± 41820
LPS (20 µg)	49571 ± 34632	83618 ± 44002

Values represent the mean cpms ± SD derived from 75 male and female control CD-1 mice used in five 90-day studies. The means for each parameter were significantly different ($p < 0.05$) among the five experiments.

TABLE 35
Control Values for Humoral Immunity as Measured by Spleen IgM AFC and Hemagglutination in Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Male		Female	
	Day 4	Day 5	Day 4	Day 5
Spleen Weight	189 ± 61	177 ± 38	196 ± 44	183 ± 55*
Spleen Cell No. (x 10^8)	1.67 ± 0.59	1.54 ± 0.44	1.68 ± 0.52	1.65 ± 0.52
IgM AFC/ Spleen (x 10^{-5})	3.15 ± 1.53	1.64 ± 0.94	3.54 ± 1.47	1.92 ± 0.73
IgM AFC/10^6 Spleen Cells	1851 ± 488	1016 ± 401	2114 ± 737	1200 ± 412
Hemagglutination Titer (Log_2)	9.39 ± 0.89		9.63 ± 1.0	

Values represent the mean ± SD derived from 70 female and male CD-1 mice used in six subchronic 90-day studies. Hemagglutination titers were derived from 48 mice used in four subchronic 90-day studies. The assays were carried out as described in Methods. The means for each parameter were significantly different ($p < 0.05$) among the experiments, except where indicated by an asterisk.

spleen cells and also as AFC/spleen. Historical control data for IgM AFC from six 90-day studies are shown in Table 35.

Hemagglutination: Mice were immunized intraperitoneally with 1 x 10^9 sRBC on day 0. On day 7, blood was collected into 3.2% sodium citrate as previously described. A sample of the plasma was removed and heat inactivated at 56°C for 30 min. Serial 1:1 dilutions were made in phosphate buffered saline in round bottom 96-well microtiter plates to a final volume of 0.1 ml. To each well was added 0.1 ml of a 0.5% sRBC solution. The plates were covered and placed in a 37°C humidified incubator. Two hr later, the plates were removed and placed on a magnifying mirror to determine at which serum dilution no agglutination occurs. The historical control data from four 90-day studies evaluating hemagglutination are shown in Table 35.

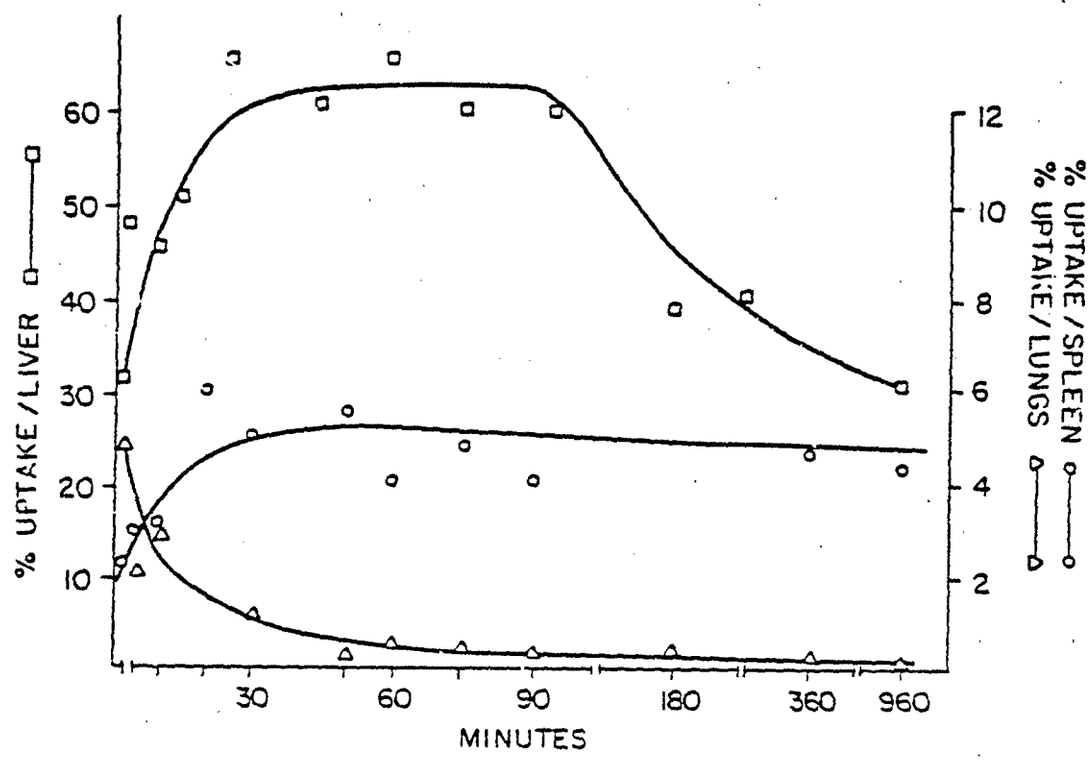
Macrophage Function

Functional Ability of the Reticuloendothelial System (RES): Five ml of freshly drawn sRBC (5 x 10^9 cells/ml) were radiolabeled with 1 mCi of sodium chromate-51 (New England Nuclear) in a 37°C shaker bath for 30 min. After chromation, the sRBC were washed with Alsever's solution until the supernatant was virtually radioactivity free. Unlabeled sRBC (5 x 10^9/ml) were added to the labeled cells until the hematocrit was approximately 12%. The resulting cpms were 200,000/0.1 ml. The sRBC were refrigerated and used the following day. Before use, the cells were washed to remove any free chromium which was release overnight, and resuspended in phosphate buffered saline (PBS).

Mice used for evaluation of RES activity were weighed and placed in shoe-box cages maintained at 39°C. At 0 time, 0.1 ml of labeled particle/10 g body weight was injected intravenously. Ten µl blood samples were taken from the tip of the tail at 2, 4, 6, 8, 10, and 15 min. At 60 min, mice were sacrificed by decapitation and selected organs collected. The blood samples were put into 1 ml of distilled water and radioassayed. Blood clearance was expressed as the phagocytic index, which was determined by the slope of the clearance curve.

Figure 28 shows the time course of particle uptake from the selected organs. For a routine study, mice were sacrificed at the plateau period (60 min after injection). Liver, spleen, lungs, thymus, and kidneys were removed, weighed, and counted in a gamma counter. Organ distribution was expressed as

FIGURE 28



The time course of particle uptake in liver, spleen, and lung. See Methods for procedure.

percent organ uptake and cpms/mg tissue (specific activity). Control values from six 90-day studies are given in Table 36.

TABLE 36

Control Values for the Functional Activity of the RES as Measured by Vascular and Organ Clearance of Cr-51 Labeled sRBC in Male and Female CD-1 Mice Used in 90-Day Studies

Parameter	Male		Female	
	% Uptake	cpm/mg	% Uptake	cpm/mg
Phagocytic Index	0.10 ± 0.05		0.11 ± 0.07	
Liver	49.6 ± 11.1	217 ± 66	57.6 ± 10.6	247 ± 69
Spleen	8.81 ± 4.08	552 ± 299	10.6 ± 6.2	493 ± 332
Lung	1.31 ± 1.11	43 ± 46	1.27 ± 0.93*	35 ± 28
Thymus	.006 ± .002	4 ± 3*	.010 ± .007	6 ± 6
Kidney	2.29 ± 1.10*	30 ± 17	2.27 ± 0.79	36 ± 13

Values represent the mean ± SD derived from 95 control CD-1 mice used in six 90-day studies. The means for each parameter were significantly different ($p < 0.05$) among the six experiments, except where indicated by an asterisk.

Recruitability of PEC: Mice were injected intraperitoneally with 1 ml of 10% Brewer's thioglycolate on day 0. On day 5, the mice were sacrificed by cervical dislocation, and the peritoneal cavity was flushed with 10 ml of minimum essential medium (MEM). The cells were centrifuged at 300 xg for 10 min and then suspended in fresh MEM. A sample was removed and enumerated on a Coulter counter in the presence of a lysing agent to determine the number of PEC recruited.

Phagocytic Activity of PEC: PEC were collected, resuspended, and counted as described above. Cell concentrations were adjusted to 2×10^5/ml and 1 ml samples added to each well of a 24-well Costar dish. Plates were incubated for one hr in a humidified CO_2 incubator (37°C). The medium was decanted and the adherent cells were washed with MEM. To each well was added 5 µl of chromated sRBC (~100,000 cpm) prepared as described above, which had been opsonized with mouse IgG. The adhered PEC and ^{51}Cr sRBC were incubated for 10, 20, 30, and 45 min. The plates were then washed once with distilled water and then twice with medium to remove any adhered sRBC. One N NaOH (1 ml) was

then added to each well to dissolve the PEC and then the contents of the wells were counted in a gamma counter.

Adherence of PEC: PEC were collected, resuspended, and counted as described previously. Cell concentrations were adjusted to 2 x 10^5/ml and 1 ml samples were placed in each well of a 24-well Costar plate. The plates were incubated for 18-24 hr in a 37°C humidified CO_2 incubator. The cells were washed extensively, scraped from the plates, and counted on a Coulter counter.

Chemotaxis of PEC: Chemotaxis of PEC was measured in modified blind well Boyden chambers using 13 mm Sartorius membrane filters as described by Haskin et al (1981). Varying numbers of cells (1 to 2 x 10^6 cells/ml) were placed in the upper portion of the chamber, with vehicle or chemoattractant (endotoxin activated mouse serum) in the lower portion. After incubation at 37°C for 4 hr, the filters were removed, rinsed in methanol, and fixed in formalin until stained. They were then placed on glass slides, stained with hematoxylin, rinsed, dipped in 0.1% NH_4OH for 1 min, and rinsed in distilled water. After processing through 95% ethanol, absolute ethanol, and isopropanol, the filters were cleared in xylene, mounted on glass slides, and dried. Using a Nikon microscope interfaced with an Artec Model 980 Image Analyzer, 20 microgrid fields were counted.

Historical control values for PEC parameters are given in Table 37.

TABLE 37
Control Values for PEC Parameters in CD-1 Mice Used in 90-Day Studies

Parameter	Male	Female
Recruitable PEC (x 10^{-7})	1.0 ± .11	2.0 ± .17
Adherent PEC (x 10^{-5})	2.6 ± .08	2.6 ± .06
Chemotaxis (cells/field)		
No Chemoattractant	22.6 ± 6	26.6 ± 6
EAMS 1:10	167 ± 27	68 ± 10
Phagocytosis of Ab-^{51}Cr sRBC (cpm)		
10 minutes	596 ± 99	354 ± 58
20 minutes	1086 ± 182	449 ± 66
30 minutes	1420 ± 216	604 ± 83
45 minutes	1693 ± 233	745 ± 112

Values represent the mean ± SD of 32 male and female CD-1 mice, derived from three 90-day studies. EAMS = endotoxin activated mouse serum, Ab-^{51}Cr sRBC= ^{51}Cr labeled sRBC opsonized with mouse IgG.

Statistical Evaluation

If a one way analysis of variance of the means showed study to study differences, a Duncan's Multiple Range Test was performed (Sall, 1979). Values which differ from study to study at $p < 0.05$ were considered statistically significant. Each of the values represents the mean ± SE.

DISCUSSION

We have chosen to approach the problem of evaluating the effects of chemicals on the lymphoreticular system from a toxicological viewpoint. Acute, 14- and 90-day studies were utilized for each chemical. The acute study was used to aid in selecting doses for subsequent studies and suggesting possible toxicological target sites. In the 90-day studies, both male and female mice were used. The outbred CD-1 mouse was our animal of choice and, as an outbred strain, the increased animal-to-animal variation was acceptable. Due to the random genetic background of the mice, many of the parameters monitored did not fall into a normally distributed response. Most notable is the variation in the number of AFC responding to sRBC in control animals. There appear to be at least three distinct populations of animals: low responders, medium responders, and high responders. In an attempt to circumvent this problem, a large number of mice was used for each treatment dose and for each parameter evaluated.

The effects of a chemical on the immune response were not studied independently, but along with a multitude of standard toxicological parameters. This results in a more complete overview of the chemical's effect. In evaluating the effects of a compound, specifically on the immune system, not just one, but several assays were used in assessing humoral immunity, cell-mediated immunity, and macrophage function.

It has been our experience that historical controls can only be used as a guide. As repeatedly pointed out in this paper, experiments using the same strain of mice, obtained from the same supplier, and handled in an identical manner by the same people results in control values which will differ significantly from experiment to experiment at the $p < 0.05$ level as determined by a one way analysis of variance. Thus, historical controls are only beneficial in establishing a data base. When comparing data for safety assessment or evaluation of drug effects, one must compare the treatment groups to the

appropriate concurrent controls used in the actual study.

Since the assays outlined here can be used to evaluate the status of the immune system in a manner which is quantifiable and reproducible, the assessment of the immunotoxicity of chemicals can be easily incorporated into routine toxicity testing. These methods have been used in this laboratory to evaluate the toxicity and immunotoxicity of some halogenated hydrocarbons found to be drinking water contaminants (Tucker et al., 1982; Sanders et al., 1982; Munson et al., 1982). The following four reports use these same approaches to assess the general toxicity and immunotoxicity of two other drinking water contaminants, trans-1,2-dichloroethylene (DCE) and 1,1,2-trichloroethane (TCE).

ACKNOWLEDGMENTS

This work was supported by grants from the Environmental Protection Agency (...06481010), the U.S. Army Medical Research and Development Command (DAMD 1778C8083), and the National Institute of Environmental Health Sciences (1T32ES07087).

B. General Toxicology of Trichloroethylene
ABSTRACT

The purpose of the study presented here was to evaluate the acute and subchronic toxicology of trichloroethylene (TCE) in the mouse. After determination of the LD_{50} by the oral route, a 14-day range-finding study was done in male CD-1 mice in which TCE was administered daily by gavage at 24 and 240 mg/kg. A drinking water study was designed based on this data, in which four concentrations of TCE were used, and mice of both sexes were exposed for four and six months. The data obtained in this study consisted of body and organ weights, hematology, clinical chemistries, and hepatic microsomal enzyme activities. There was a decreased weight gain at the highest dose, which could be attributed to a decrease in fluid consumption. Perhaps the most significant effect attributable to TCE was an increase in liver weight of approximately 20% in both sexes. The data presented consists of a toxicological data profile on subchronic, oral TCE exposure. Although the effects of TCE were largely negative, it does serve as background for the immunotoxicological effects to be presented in the following report.

Running Title: Toxicology of Trichloroethylene

INTRODUCTION

Our laboratory is presently attempting to define the effects of selected environmental chemicals on host immunocompetence. Since it is only recently that immunotoxicology has become an area of interest, development of methods in this field is of utmost importance. Furthermore, correlation of general toxicological effects with immunosuppressive effects is essential in order that the results be more clearly understood and kept in perspective.

One of the compounds we have studied is trichloroethylene (also known as TCE or trichloroethene), a widely used solvent which has been detected in water, air, food, and human tissues (McConnell et al., 1975, Pearson and McConnell, 1975). Manifestations of acute toxicity to TCE include central nervous system depression and liver damage (Stewart et al., 1970; Defalque, 1961; Waters et al., 1977). There is a general lack of information concerning chronic effects, other than the very important observation that TCE is hepatocarcinogenic in the mouse (NCI, 1976).

The present paper is the second in a series of reports dealing with immunotoxicological investigations in the mouse and concerns general toxicological effects of TCE. Effects on humoral and cell mediated immunity, and effects on the macrophage following subchronic oral exposure to TCE will be presented in the following paper. Investigations on five other chlorinated hydrocarbons found in the drinking water are under way and will be reported using the same approach. A known immunosuppressive agent, dexamethasone, will be reported in this series for comparative purposes.

MATERIALS AND METHODS

Animals and Housing

Weanling mice (CD-1, an ICR outbred albino) were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. Upon arrival, all mice were quarantined for a period of one week prior to exposure. Mice were housed five per cage in plastic shoebox cages with sawdust bedding and maintained on Agway Lab Chow ad libitum. Each mouse was individually tagged by earpunching. Room temperature was maintained at 70-75°F and relative humidity at 40-60%. The light-dark cycle was maintained on 12-hour intervals.

Trichloroethylene (TCE), certified ACS, lot #713839, was obtained from J.T. Baker Chemical Co., Phillipsburg, N.J. and contained 0.004% diisopropylamine as preservative.

Trichloroethylene administered ad libitum in the drinking water was dissolved in a 1% emulphor (polyethoxylated vegetable oil, GAF 620) solution in deionized water at the indicated concentrations. Drinking water solutions were maintained at room temperature in amber-colored bottles with sipper spouts and were changed twice weekly. Less than 20% of the TCE at the 1.0, 2.5, and 5.0 mg/ml concentrations was lost during the three and four days in the water bottles as measured by GLC with head space analysis. In the case of 0.1 mg/ml, up to 45% was lost over a four-day period. Preliminary studies using different concentrations of emulphor indicated that a 1% solution would maintain the TCE in solution while causing minimal effects. A naive group (deionized water) was nevertheless included in the subchronic study. Solutions

for gavage were prepared fresh daily in 10% emulphor and appropriate concentrations administered in a volume of 0.1 ml/g body weight to achieve the desired dose.

Animal Necropsy

Animals were anesthetized with chloroform, and blood was collected immediately by cardiac puncture. Gross pathological examinations were performed on all mice. The organs (brain, liver, spleen, lungs, thymus, kidneys and testes) were then removed, trimmed and weighed.

Urinalysis

Urine collected during cardiac puncture was assayed using Labstix reagent strips from Ames Co., Elkhart, Indiana.

Hematology

Blood samples were taken in 3.2% sodium citrate. Leukocyte, erythrocyte, and platelet counts were performed on a Coulter Counter, Model ZBI. Hematocrits were performed with microhematocrit equipment and hemoglobins determined as cyanomethemoglobin. Leukocyte differentials were evaluated using the standard Wright's-Giemsa staining procedure. Bone marrow cells were flushed from the femur in α MEM medium with 5% fetal calf serum and enumerated on the Coulter Counter.

The plasma from the blood samples was assayed for extrinsic activity by prothrombin time. Reagents for this assay were obtained from General Diagnostics. Fibrinogen levels were determined by the kinetic method, using reagents from Dade Diagnostics, Inc. Miami, FL.

For clinical chemistry studies, additional blood samples were drawn by cardiac puncture from animals used for studies on humoral immunity (described in the following report) and allowed to clot. The sera from these samples were loaded onto the Abbott Bichromatic Analyzer, Model 100, and processed according to procedures described in the Abbott Operator's Manual.

Preparation and Assay of Microsomes

Livers were removed, weighed, rinsed, and homogenized at $4^{o}C$ in 4 volumes of 0.15 M KCl containing 0.1 M potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 9000 x g for 20 minutes and the supernatant recentrifuged at 100,000 x g for 1 hour in a Beckman Model L5-50 ultracentrifuge. The microsomes in the pellet were resuspended in 0.05 M potassium phosphate buffer, pH 7.4 (0.5 g of liver/ml), and aliquots taken for immediate study.

Microsomal protein was assayed by the method of Lowry (1951). The cytochrome P-450 content was determined from the reduced CO vs. reduced difference spectrum, using an extinction coefficient of 91 $cm^{-1}mM^{-1}$ (Omura and Sato, 1964). Aminopyrine N-demethylase activity was determined by measuring formaldehyde production by the Nash reaction as described by Cochin and Axelrod (1959). Aniline hydroxylase activity was measured as p-aminophenol production (Imai et al., 1966).

Statistical Analysis

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's T-test was performed (Dunnett, 1964). Values which differ from vehicle control at $P < .05$ are noted in the tables with an asterisk. Each of the values in the tables is given as mean ± standard error (S.E.) of the mean.

RESULTS

Acute Study

Table 38 shows the acute toxicity data for trichloroethylene. No deaths occurred at doses up to 750 mg/kg for females and 1250 mg/kg for males, but there was 100% mortality at doses of 5500 mg/kg for females and 6000 mg/kg for males. The LD_{50}'s with 95% confidence limits are 2454 mg/kg (2040-3062) for female mice and 2402 mg/kg (2065-2771) for male mice. The slope functions of the dose-response curves for female and male mice are 1.835 and 1.587 respectively.

The deaths occurred within 24 hours of trichloroethylene administration. Other responses observed were dependent on dose and time and occurred in the following sequence: ataxia, loss of ability to stand, ruffled fur, loss of righting reflex, loss of response to tendon pressure, abdominal breathing and death. No animals that recovered from anesthesia died during the 14-day observation period. The only gross pathology observed was hyperemia of the stomach of mice dying from lethal doses of trichloroethylene. Mice sacrificed at 14 days showed no gross pathology.

TABLE 38

Acute Toxicity (mg/kg) of Trichloroethylene in CD-1 Mice

Sex	LD_{10}	LD_{50}	LD_{90}	Slope
Females (N=121)	1159 (795 - 1450)	2454 (2040 - 8592)	5197 (3939 - 8592)	1.835
Males (N=136)	1356 (972 - 1645)	2402 (2065 - 2771)	5001 (4055 - 7250)	1.587

CD-1 male and female mice were fasted for 18 hours prior to a single gavage of trichloroethylene in doses between 250-6000 mg/kg. The LD_{10}, LD_{50} and LD_{90} with corresponding 95% confidence limits were calculated according to the Log Probit Procedure using the SAS 79 computer program.

Fourteen-Day Range Finding Study

The first subchronic experiment consisted of exposure of male CD-1 mice to TCE by daily gavage for 14 days. The two doses chosen (240 and 24 mg/kg) were approximately 1/10 and 1/100 of the LD_{50} and caused no treatment-related deaths. As shown in Table 39, body weight was not significantly affected, nor were most organ weights. The only effect noted was an increased liver weight (33% greater than control) at the higher dose, which appears to be dose related. The only significant difference found in hematology was a lower hematocrit in the higher dose group (Table 40). This does not appear to be dose related and is well within our normal range.

Hepatic Microsomal Activities

As described in Table 41, a group of mice were given TCE daily by gavage for five days. The average daily dose was 2.62 g/kg. This treatment produced a significant number of deaths and a decrease in body weight. The treatment also caused an increase in microsomal protein, a decrease in aminopyrine N-demethylase activity, and an increase in aniline hydroxylase activity as compared to vehicle control.

TABLE 39

Body and Organ Weights of Male Mice Exposed to Trichloroethylene by Gavage for Fourteen Days

Weights	Trichloroethylene (mg/kg) Vehicle	24	240
(Number of Mice)	(12)	(12)	(12)
Body Weight (g)	29.7 ± 0.8	31.1 ± 0.4	30.8 ± 0.6
Brain (mg)	426 ± 9	441 ± 5	425 ± 9
(% body weight)	(1.44)	(1.42)	(1.39)
Liver (mg)	1886 ± 82	2012 ± 64	2505 ± 77*
(% body weight)	(6.34)	(6.48)	(8.14)*
Spleen (mg)	131 ± 9	146 ± 10	135 ± 8
(% body weight)	(0.44)	(0.46)	(0.44)
Lungs (mg)	190 ± 7	206 ± 9	213 ± 9
(% body weight)	(0.64)	(0.66)	(0.70)
Thymus (mg)	74 ± 5	76 ± 2	65 ± 4
(% body weight)	(0.25)	(0.24)	(0.21)
Kidneys (mg)	485 ± 15	495 ± 18	493 ± 9
(% body weight)	(1.64)	(1.59)	(1.60)
Testes (mg)	199 ± 8	198 ± 5	187 ± 7
(% body weight)	(0.67)	(0.63)	(0.61)

The vehicle in this study was 10% emulphor in distilled water. Values are mean ± S.E. with those significantly different from vehicle at $p < .05$ noted by an asterisk.

TABLE 40

Hematology of Male Mice Exposed to Trichloroethylene by Gavage for Fourteen Days

Parameter	Trichloroethylene (mg/kg) Vehicle	24	240
(Number of Mice)	(12)	(12)	(12)
Hematocrit (%)	41.5 ± 0.5	42.2 ± 0.4	39.3 ± 0.7*
Hemoglobin (g%)	12.2 ± 0.3	12.7 ± 0.3	11.4 ± 0.5
Leukocytes ($10^3/mm^3$)	5.93 ± 0.46	5.15 ± 0.44	6.15 ± 0.38
Fibrinogen (mg%)	283 ± 5	294 ± 7	272 ± 8
Prothrombin Time (sec)	8.5 ± 0.1	8.4 ± 0.1	8.5 ± 0.1
LDH (IU/L)	1024 ± 92	938 ± 64	880 ± 41
SGPT (IU/L)	85.7 ± 10.1	70.3 ± 5.4	104.5 ± 27.5
BUN (IU/L)	31.1 ± 0.8	29.1 ± 1.5	28.1 ± 0.7

LDH = Lactic dehydrogenase; SGPT = Serum glutamic pyruvic transaminase; BUN = Blood urea nitrogen. Values represent Mean ± S.E. with those differing from vehicle at $P < .05$ noted by an asterisk.

TABLE 41

Effect of Trichloroethylene Administered to Male Mice by Gavage for Five Days on Hepatic Microsomal Activites

Parameter	Vehicle	Treated
(Number of Mice)	(4)	(4)
Body Weight Change	- 3.6%	- 22.7%*
Liver Weight (g)	1.50 ± .06	1.40 ± .16
Microsomal Protein (mg/g liver)	21.8 ± 0.3	26.8 ± 1.2*
Cytochrome P-450 (nmol/mg protein)	0.59 ± .03	0.58 ± .04
Cytochrome b_5 (nmol/mg protein)	0.38 ± .01	0.37 ± .02
Aminopyrine N-demethylase (nmol/mg/min)	9.91 ± .56	5.80 ± .48*
Aniline hydroxylase (nmol/mg/min)	1.32 ± .10	1.94 ± .20*

Mice received 0.73 g/kg twice on day 0, 1.46 g/kg twice on day 1, 2.91 g/kg twice on day 3, and 1.46 g/kg on days 4 and 5. They were sacrificed on day 6 for assay. Only 4 out of 11 mice treated with TCE survived. Values represent the Mean ± S.E., those which differ from vehicle at $P < .05$ are noted by an asterisk.

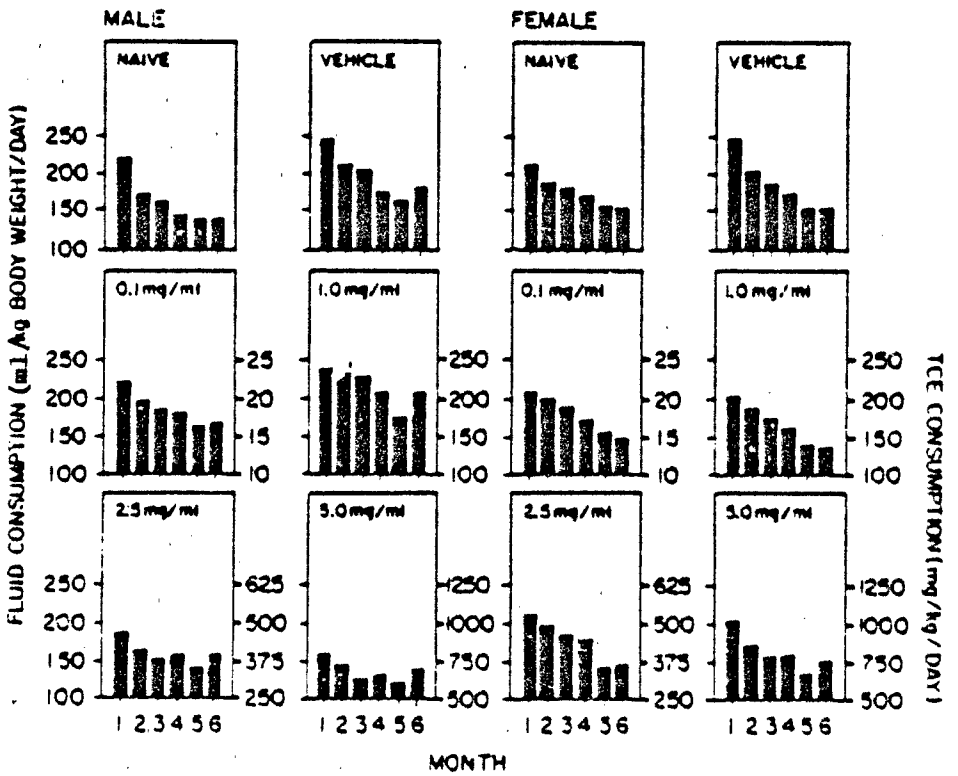
Subchronic Four- and Six-Month Studies

A study was set up using both sexes of CD-1 mice and drinking water levels of 0.1, 1.0, 2.5 and 5 mg/ml of TCE in 1% emulphor. A naive group receiving deionized water was included. There were 140 animals of each sex in the naive and in each treatment group, except for 260 in the vehicle groups. Thirty mice of each sex and treatment were selected for recording periodic weights for six months. These mice were weighed twice weekly, and fluid consumption was measured by weighing the six corresponding water bottles. The doses of TCE were then calculated, and this data is shown in Figure 29 for each month of study. Fluid consumption was decreased in the males receiving the two highest concentrations of TCE and somewhat decreased in the females. The growth chart for the males is shown in Figure 30, and that for females in Figure 31. Some of the animals were sacrificed after four months of exposure, the remainder after six months. The doses of TCE consumed as a time-weighted average at four and six months for each of the four concentrations are given in Table 42. There were no treatment-related deaths in the study.

Gross pathology revealed a few effects worth mentioning. Several livers from animals in the three highest dose groups of both sexes appeared abnormal at six months. They were

FIGURE 29

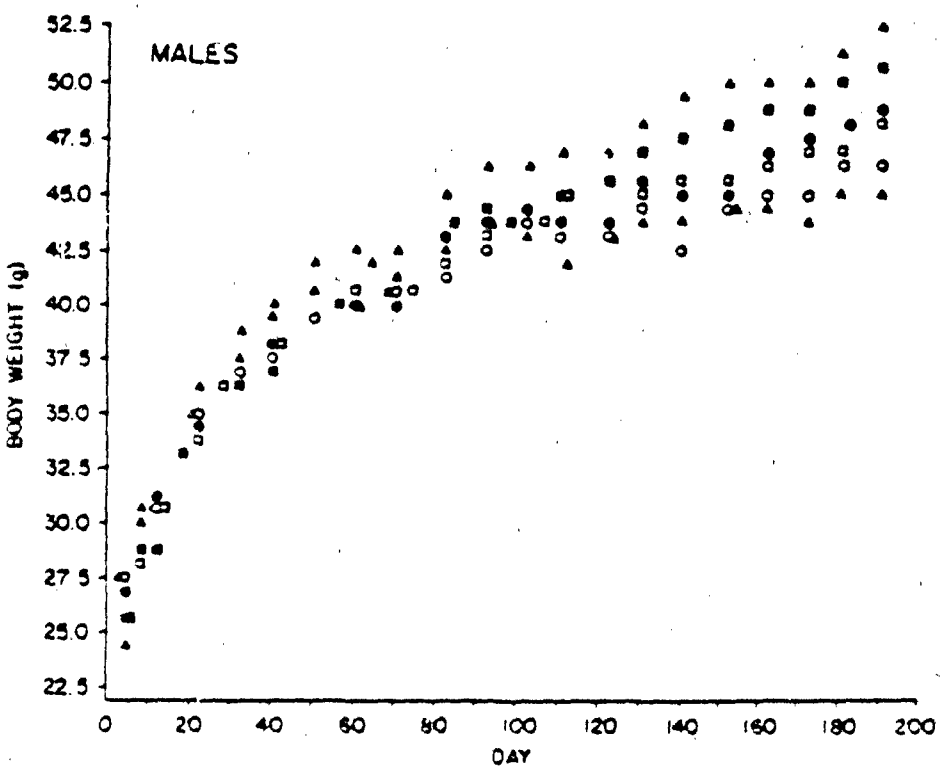
Consumption of fluid and trichloroethylene (TCE) by male and female mice.



Naive animals received deionized water; vehicle was 1% emulphor in water; the four concentrations of TCE are given in each box (0.1, 1.0, 2.5, 5.0 mg/ml). The left axis of each box is fluid consumed in ml/kg body weight/day. Based on the TCE concentration, this is translated to TCE consumed (right axis) in mg/kg body weight/day. These values are shown as the averages for each month of the study. There were 30 animals in each group.

FIGURE 30

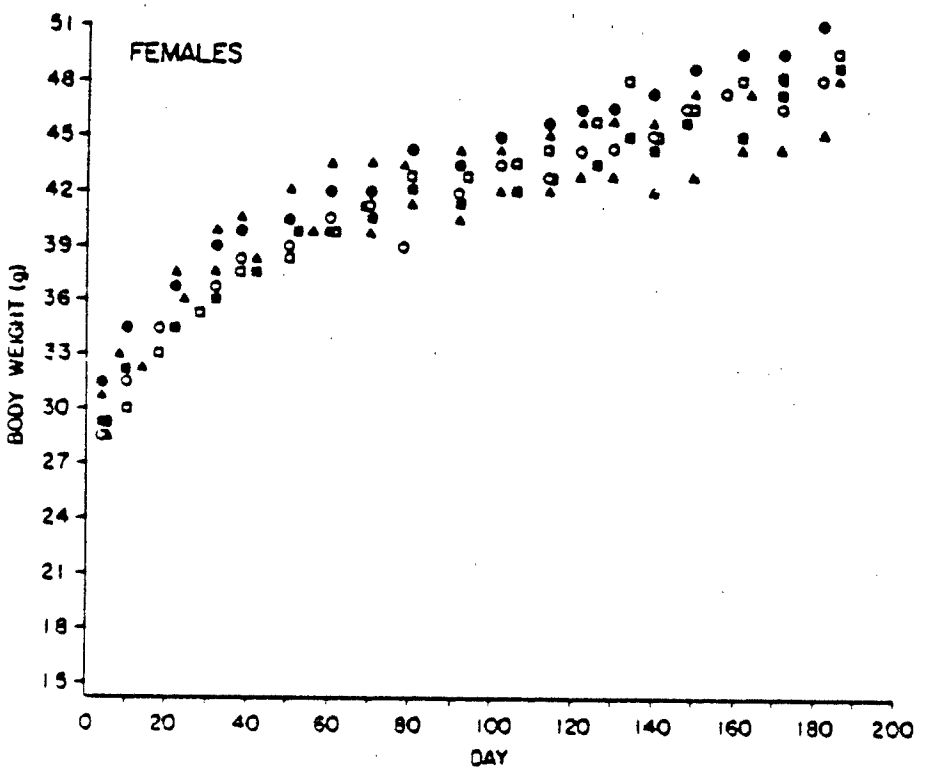
Growth chart of male CD-1 mice receiving trichloroethylene in the drinking water.



The mice received on e of the following: (■) deionized water; (□) 1% emulphor; (●) 0.1 mg/ml TCE; (○) 1.0 mg/ml TCE; (▲) 2.5 mg/ml TCE; (△) 5.0 mg/ml TCE. Each symbol represents the mean of 30 mice.

FIGURE 31

Growth chart of female CD-1 mice receiving tricholoroethylene in the drinking water



The mice received one of the following: (■) deionized water; (□) 1% emulphor; (●) 0.1 mg/ml TCE; (○) 1.0 mg/ml TCE; (▲) 2.5 mg/ml TCE; (△) 5.0 mg/ml TCE. Each symbol represents the mean of 30 mice.

TABLE 42

Average Daily Doses (mg/kg) of Trichloroethylene (TCE) Consumed at Four and Six Months

	TCE Concentration (mg/ml)			
	0.1	1.0	2.5	5.0
Males:				
Four Months	18.9	219.6	397.2	664.5
Six Months	18.4	216.7	393.0	660.2
Females:				
Four Months	18.7	201.4	457.4	809.3
Six Months	17.9	193.0	437.1	793.3

The doses were calculated from the fluid consumption and body weights of the eight groups of males and females and are time-weighted averages for the two periods of time. The complete data are shown in Figure 29.

either pale, granular or had white spots, and one nodule was found (high dose male). This animal also had an enlargement of one kidney, as did one male in the 1 mg/ml group. Several males in the 1 and 5 ml groups had evidence of gastrointestinal problems, consisting of gas pockets in the intestinal coating and blood in the intestines. One high-dose male had a twisted spleen with adhesions. A few aniamls with hyperemic lungs were observed among the higher doses. Of the observed abnormalities, only the pale, blotched livers were observed in naive and vehicle groups; however, neither of these groups showed evidence of granular livers or had any other organ abnormalities.

The average body weight was significantly lower in the males receiving the highest dose at both four and six months. Enlarged livers (as percent body weight) were observed in males and females at the three higher doses at both times, although the difference was not significant in the females at six months (Tables 43-46). The apparent increase in brain weight in treated females at 4 months is likely due to a low value in the vehicle group, since the naive value is also high. A significant increase in kidney weight occurred at the highest dose in males at six months and females at both four and six months. Noteworthy hematology differences (Tables 47-50) include a decreased erythrocyte count in the males at four and six months; decreased leukocyte counts,

TABLE 43

Body and Organ Weights of Male Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(25)	(15)	(15)	(14)	(15)
Body Weight (g)	47.5 ± 1.3	46.8 ± 1.4	46.4 ± 1.1	44.9 ± 1.8	46.1 ± 1.9	42.3 ± 0.9*
Brain (mg)	492.3 ± 7.7	479.2 ± 8.6	486.2 ± 1.23	463.4 ± 9.6	490.4 ± 8.3	461.3 ± 7.9
(% body weight)	(1.05)	(1.04)	(1.05)	(1.05)	(1.08)	(1.10)
Liver (mg)	2160 ± 83*	2488 ± 72	2444 ± 58	2647 ± 145	2982 ± 148*	2774 ± 96
(% body weight)	(4.55)*	(5.33)	(5.29)	(5.86)*	(6.44)*	(6.53)*
Spleen (mg)	198 ± 18	184 ± 8	170 ± 13	166 ± 5	189 ± 17	159 ± 8
(% body weight)	(0.40)	(0.42)	(0.37)	(0.37)	(0.41)	(0.38)
Lungs (mg)	279 ± 12	280 ± 10	267 ± 10	254 ± 6	268 ± 12	258 ± 13
(% body weight)	(0.59)	(0.61)	(0.58)	(0.57)	(0.59)	(0.61)
Thymus (mg)	78 ± 7	89 ± 8	84 ± 8	71 ± 10	82 ± 10	62 ± 7*
(% body weight)	(0.16)	(0.19)	(0.18)	(0.15)	(0.18)	(0.15)
Kidneys (mg)	663 ± 20	722 ± 22	703 ± 21	699 ± 23	752 ± 30	705 ± 26
(% body weight)	(1.40)*	(1.56)	(1.52)	(1.58)	(1.64)	(1.67)
Testes (mg)	296 ± 9	280 ± 9	290 ± 12	272 ± 7	271 ± 8	267 ± 9
(% body weight)	(0.63)	(0.61)	(0.63)	(0.62)	(0.60)	(0.63)

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 4.

TABLE 44

Body and Organ Weights of Male Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(13)	(23)	(15)	(15)	(15)	(20)
Body Weight (g)	49.5 ± 1.6	49.4 ± 1.7	49.5 ± 1.6	45.9 ± 1.6	51.0 ± 1.6	44.6 ± 0.8*
Brain (mg)	496 ± 9	477 ± 7	500 ± 10	438 ± 8	498 ± 9	492 ± 7
(% body weight)	(1.01)	(0.99)	(1.02)	(1.0P)	(0.99)	(1.11)
Liver (mg)	2533 ± 101	2699 ± 110	2746 ± 110	2897 ± 159	3201 ± 181*	2745 ± 77
(% body weight)	(5.13)	(5.48)	(5.54)	(6.29)*	(6.26)	(6.18)*
Spleen (mg)	208 ± 22	210 ± 19	184 ± 14	174 ± 13	177 ± 12	209 ± 31
(% body weight)	(0.42)	(0.44)	(0.37)	(0.38)	(0.35)	(0.48)
Lungs (mg)	276 ± 6	266 ± 7	280 ± 13	265 ± 9	287 ± 12	283 ± 12
(% body weight)	(0.56)	(0.55)	(0.57)	(0.58)	(0.57)	(0.64)
Thymus (mg)	101 ± 11	87 ± 7	96 ± 7	88 ± 7	97 ± 9	68 ± 7
(% body weight)	(0.20)	(0.18)	(0.19)	(0.19)	(0.19)	(0.16)
Kidneys (mg)	740 ± 30	762 ± 17	762 ± 16	793 ± 25	813 ± 22	774 ± 15
(% body weight)	(1.53)	(1.58)	(1.55)	(1.74)*	(1.61)	(1.73)*
Testes (mg)	290 ± 9	275 ± 10	281 ± 10	273 ± 10	299 ± 19	295 ± 11
(% body weight)	(0.59)	(0.57)	(0.58)	(0.60)	(0.59)	(0.66)

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 4.

TABLE 45

Body and Organ Weights of Female Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Trichloroethylene (mg/ml)					
	Naive	Vehicle	0.1	1.0	2.5	5.0
(Number of Mice)	(14)	(26)	(15)	(15)	(14)	(15)
Body Weight (g)	40.6 ± 2.1*	36.0 ± 0.6	39.3 ± 1.3	42.1 ± 1.7*	39.3 ± 1.6	39.3 ± 1.1
Brain (mg)	487 ± 6*	458 ± 8	492 ± 7*	492 ± 5*	484 ± 9*	481 ± 7*
(% body weight)	(1.24)	(1.28)	(1.27)	(1.19)	(1.26)	(1.24)
Liver (mg)	1800 ± 103	1840 ± 64	1960 ± 76	2090 ± 50*	2065 ± 69*	2267 ± 107*
(% body weight)	(4.46)	(5.11)	(5.02)	(5.03)	(5.30)	(5.76)*
Spleen (mg)	200 ± 16	186 ± 7	206 ± 18	181 ± 13	187 ± 13	192 ± 16
(% body weight)	(0.50)	(0.52)	(0.53)	(0.44)	(0.48)	(0.49)
Lungs (mg)	228 ± 9	225 ± 7	257 ± 6	245 ± 9	224 ± 12	245 ± 10
(% body weight)	(0.57)	(0.63)	(0.66)	(0.59)	(0.57)	(0.63)
Thymus (mg)	94 ± 9*	76 ± 5	92 ± 8	89 ± 7	78 ± 7	92 ± 7
(% body weight)	(0.23)	(0.21)	(0.24)	(0.21)	(0.20)	(0.24)
Kidneys (mg)	422 ± 12	437 ± 12	478 ± 15	473 ± 14	471 ± 20	524 ± 14*
(% body weight)	(0.23)	(1.22)	(1.23)	(1.14)	(1.20)	(1.34)*

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 4.

TABLE 46

Body and Organ Weights of Female Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(23)	(15)	(15)	(15)	(15)
Body Weight (g)	40.0 ± 1.4	44.5 ± 1.7	46.0 ± 2.4	45.6 ± 1.4	43.9 ± 1.8	39.2 ± 1.5
Brain (mg)	486 ± 11	494 ± 7	488 ± 11	495 ± 8	477 ± 9	488 ± 9
(% body weight)	(1.24)	(1.14)	(1.10)	(1.10)	(1.11)	(1.26)
Liver (mg)	1972 ± 69*	2258 ± 88	2218 ± 70	2299 ± 100	2406 ± 98	2346 ± 76
(% body weight)	(4.97)	(5.12)	(4.94)	(5.04)	(5.50)	(6.02)
Spleen (mg)	187 ± 18	188 ± 10	205 ± 9	175 ± 12	202 ± 13	194 ± 12
(% body weight)	(0.47)	(0.44)	(0.46)	(0.39)	(0.47)	(0.50)
Lungs (mg)	232 ± 7	234 ± 8	251 ± 11	227 ± 7	215 ± 9	229 ± 11
(% body weight)	(0.58)	(0.54)	(0.56)	(0.50)	(0.50)	(0.59)
Thymus (mg)	88 ± 8	98 ± 8	105 ± 6	95 ± 9	91 ± 8	80 ± 9
(% body weight)	(0.22)	(0.22)	(0.23)	(0.21)	(0.21)	(0.21)
Kidneys (mg)	448 ± 13	491 ± 15	505 ± 17	492 ± 21	484 ± 18	517 ± 22
(% body weight)	(1.13)	(1.12)	(1.13)	(1.09)	(1.13)	(1.33)*

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 4.

TABLE 47

Hematology of Male Mice Exposed to Trichlorethylene
in Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(12)	(22)	(15)	(15)	(15)	(19)
Hematocrit (%)	41.4 ± 0.7	41.2 ± 0.6	42.2 ± 0.8	42.5 ± 0.6	41.2 ± 0.7	39.7 ± 0.9
Hemoglobin (g%)	9.8 ± 0.5	10.6 ± 0.5	12.3 ± 0.4*	12.4 ± 0.3*	12.3 ± 0.2*	11.1 ± 0.3
Erythrocytes ($10^6/mm^3$)	9.45 ± 0.37	8.83 ± 0.28	8.95 ± 0.23	8.37 ± 0.32	8.28 ± 0.13	7.91 ± 0.15*
Leukocytes ($10^3/mm^3$)	7.50 ± 0.90	6.83 ± 0.64	5.96 ± 0.63*	6.74 ± 0.49	5.43 ± 0.36*	8.22 ± 0.88
Platelets ($10^5/mm^3$)	2.32 ± 0.18	2.02 ± 0.11	2.28 ± 0.12	2.58 ± 0.10*	2.53 ± 0.14*	2.75 ± 0.18*
Fibrinogen (mg%)	305 ± 14	309 ± 10	326 ± 15	374 ± 8*	344 ± 12*	403 ± 14*
Prothrombin Time (sec)	9.4 ± 0.3	8.8 ± 0.3	9.1 ± 0.4	9.8 ± 0.3	10.2 ± 0.6	8.3 ± 0.3
Bone Marrow (10^7 cells/femur)	2.06 ± 0.17	2.05 ± 0.10	2.23 ± 0.15	2.72 ± 0.28	2.41 ± 0.27	2.47 ± 0.12

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 48

Hematology of Male Mice Exposed to Trichloroethylene
in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(25)	(15)	(15)	(13)	(15)
Hematocrit (%)	38.8 ± 0.5	39.2 ± 0.4	39.8 ± 0.6	40.1 ± 0.4	39.4 ± 0.8	39.7 ± 0.7
Hemoglobin (g%)	11.7 ± 0.2	11.7 ± 0.2	11.7 ± 0.2	11.5 ± 0.2	11.6 ± 0.3	11.2 ± 0.3
Erythrocytes (10^6/mm^3)	7.95 ± 0.21	7.89 ± 0.18	8.10 ± 0.18	7.84 ± 0.26	7.66 ± 0.21	6.94 ± 0.20*
Leukocytes (10^3/mm^3)	7.49 ± 0.73*	5.83 ± 0.27	6.81 ± 0.47	7.22 ± 0.57*	6.60 ± 0.47	6.46 ± 0.64
Platelets (10^5/mm^3)	[illegible] ± 0.14	2.87 ± 0.11	2.75 ± 0.10	2.67 ± 0.16	2.55 ± 0.16	2.51 ± 0.12
Fibrinogen (mg%)	364 ± 19*	268 ± 6	274 ± 7	318 ± 14*	337 ± 17*	323 ± 23*
Prothrombin Time (sec)	9.9 ± 0.2*	8.5 ± 0.1	8.9 ± 0.2*	8.7 ± 0.1	8.4 ± 0.1	9.1 ± 0.1*
Bone Marrow (10^7 cells/femur)	2.12 ± 0.15	2.46 ± 0.12	2.49 ± 0.24	2.29 ± 0.12	2.75 ± 0.18	2.70 ± 0.16

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 49

Hematology of Female Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(13)	(26)	(15)	(15)	(13)	(14)
Hematocrit (%)	38.7 ± 0.6	40.1 ± 0.2	40.7 ± 0.8	40.1 ± 0.7	41.5 ± 0.6*	39.3 ± 0.4
Hemoglobin (g%)	11.9 ± 0.2	11.7 ± 0.2	12.3 ± 0.2*	11.8 ± 0.2	12.2 ± 0.2	11.4 ± 0.2
Erythrocytes ($10^6/mm^3$)			[NOT DONE]			
Leukocytes ($10^3/mm^3$)	7.50 ± 0.50	7.52 ± 0.36	6.62 ± 0.35	6.38 ± 0.34*	6.37 ± 0.34*	6.11 ± 0.44*
Platelets ($10^5/mm^3$)	2.23 ± 0.14	2.21 ± 0.12	2.14 ± 0.12	2.34 ± 0.12	2.20 ± 0.13	2.17 ± 0.14
Fibrinogen (mg%)	248 ± 15	210 ± 7	203 ± 6	212 ± 4	231 ± 7	208 ± 7
Prothrombin Time (sec)	9.0 ± 0.1	9.2 ± 0.4	10.1 ± 0.6	10.4 ± 0.6	10.6 ± 0.7	10.2 ± 0.5
Bone Marrow (10^7 cells/femur)	1.78 ± 0.07	1.75 ± 0.11	1.82 ± 0.14	2.08 ± 0.07	2.02 ± 0.10	1.84 ± 0.11

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 50

Hematology of Female Mice Exposed to Trichlorethylene
in the Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(23)	(14)	(14)	(15)	(15)
Hematocrit (%)	40.5 ± 0.4	39.0 ± 0.5	40.3 ± 0.4	40.5 ± 0.4	39.3 ± 0.8	40.1 ± 0.8
Hemoglobin (g%)	12.2 ± 0.6	11.2 ± 0.4	11.4 ± 0.6	11.1 ± 0.4	10.6 ± 0.5	10.6 ± 0.4
Erythrocytes (10^6/mm^3)	9.30 ± 0.5	8.38 ± 0.26	7.97 ± 0.28	8.06 ± 0.36	7.45 ± 0.29	8.19 ± 0.33
Leukocytes (10^3/mm^3)	6.70 ± 0.49	6.03 ± 0.51	5.96 ± 0.67	5.15 ± 0.41	5.[illegible] ± 0.31	6.19 ± 0.49
Platelets (10^5/mm^3)	1.82 ± 0.11	2.04 ± 0.11	2.17 ± 0.18	2.17 ± 0.12	2.21 ± 0.20	2.26 ± 0.11
Fibrinogen (mg%)	264 ± 9	257 ± 10	288 ± 11*	262 ± 13	273 ± 14	316 ± 14*
Prothrombin Time (sec)	8.3 ± 0.1	8.2 ± 0.1	7.3 ± 0.1*	7.0 ± 0.1*	7.8 ± 0.2*	7.3 ± 0.2*
Bone Marrow (10^7 cells/femur)	2.10 ± 0.14	2.46 ± 0.17	2.46 ± 0.42	2.33 ± 0.19	2.13 ± 0.28	2.49 ± 0.39

Values in all tables are given as the mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

particularly in the females at four months; and altered coagulation values consisting of increased fibrinogen in males at both times and prolonged prothrombin time in females at six months. As shown in Table 51, there were no treatment-related effects on the types of white cells in peripheral blood. No treatment-related effects could be detected on urinalysis (protein, glucose, pH, ketones, bilirubin or blood content). Clinical chemistry values are shown in Tables 52 - 55. A few values were significantly different from vehicle control, but there are no consistent trends. Not enough historical controls have accumulated to know if these values are within normal range or not. Hepatic microsomal activities which were measured are shown in Tables 56-59. Aniline hydroxylase activity and cytochrome P-450 were increased in females at four months. These were the only treatment differences observed.

TABLE 51

Differential Cell Counts of Mice Exposed to Trichloroethylene (TCE) in the Drinking Water for Four or Six Months

	N	Lymph	PMN	Mono	Eosi
Males (Four Months)					
Naive	15	71.1 ± 1.2	22.9 ± 1.6	1.6 ± 0.3	4.2 ±
Vehicle	22	70.8 ± 2.2	23.7 ± 2.5	0.8 ± 0.3	4.7 ±
0.1 mg/ml	15	68.3 ± 2.6	27.5 ± 2.5	1.7 ± 0.4*	2.5 ±
1.0 mg/ml	15	72.5 ± 1.6	23.8 ± 1.7	0.5 ± 0.1	3.3 ±
2.5 mg/ml	13	71.1 ± 1.9	23.0 ± 1.5	0.8 ± 0.2	5.1 ±
5.0 mg/ml	15	69.9 ± 1.9	24.9 ± 1.5	0.9 ± 0.3	5.0 ±
Males (Six Months)					
Naive	14	69.0 ± 4.3	27.0 ± 4.0	1.4 ± 0.4*	1.9 ±
Vehicle	22	70.3 ± 3.1	28.4 ± 3.0	0.4 ± 0.2	1.4 ±
0.1 mg/ml	15	74.1 ± 3.2	25.2 ± 3.1	0.3 ± 0.1	0.9 ±
1.0 mg/ml	15	65.1 ± 3.1	33.3 ± 3.4	0.4 ± 0.2	1.2 ±
2.5 mg/ml	15	74.9 ± 3.1	23.9 ± 3.0	0.4 ± 0.1	1.5 ±
5.0 mg/ml	17	71.8 ± 2.0	25.1 ± 1.9	0.1 ± 0.1	2.9 ±
Females (Four Months)					
Naive	15	77.7 ± 1.5	17.2 ± 1.5	0.5 ± 0.2	4.6 ±
Vehicle	23	79.4 ± 1.8	17.7 ± 1.4	0.3 ± 0.1	3.0 ±
0.1 mg/ml	15	76.1 ± 2.4	20.3 ± 2.0	0.8 ± 0.4	2.9 ±
1.0 mg/ml	14	73.1 ± 2.1	22.1 ± 1.9	0.3 ± 0.2	4.6 ±
2.5 mg/ml	15	77.1 ± 1.3	20.6 ± 1.1	0.3 ± 0.1	2.0 ±
5.0 mg/ml	14	78.9 ± 1.5	18.4 ± 1.5	0.1 ± 0.1	2.6 ±
Females (Six Months)					
Naive	15	83.3 ± 2.7	15.1 ± 2.4	0.8 ± 0.4	0.8 ±
Vehicle	23	83.9 ± 2.1	15.1 ± 2.0	0.04 < 0.1	1.0 ±
0.1 mg/ml	15	76.3 ± 2.5	22.8 ± 2.4	0.1 ± 0.1	0.8 ±
1.0 mg/ml	15	79.1 ± 3.4	19.7 ± 3.4	0.3 ± 0.2	0.9 ±
2.5 mg/ml	14	85.2 ± 2.3	13.6 ± 2.2	0.4 ± 0.2	0.7 ±
5.0 mg/ml	15	80.8 ± 2.7	17.0 ± 2.7	0.5 ± 0.3	1.7 ±

Values are averages of percent of total white cells with those values significantly different at $P < .05$ noted by an asterisk.

TABLE 52

Clinical Chemistry Values of Male Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(14)	(14)	(12)	(13)	(14)	(14)
Calcium (mg%)	11.55 ± 0.68	9.87 ± 0.33	9.11 ± 0.14	8.77 ± 0.11	9.21 ± 0.12	9.37 ± 0.16
Sodium (mEq/L)	158 ± 2	157 ± 2	161 ± 3	162 ± 3	157 ± 2	158 ± 2
Chloride (mEq/L)	107 ± 1	106 ± 1	108 ± 1	109 ± 1	103 ± 1	105 ± 1
Potassium (mEq/L)	6.41 ± 0.17	6.78 ± 0.34	6.79 ± 0.16	7.28 ± 0.37	6.58 ± 0.20	6.66 ± 0.17
Protein (g%)	7.30 ± 0.43	6.88 ± 0.33	6.28 ± 0.32	5.43 ± 0.18*	7.14 ± 0.35	7.46 ± 0.51
Glucose (mg%)	132 ± 8	124 ± 8	96 ± 6*	95 ± 4*	102 ± 4	119 ± 9
Cholesterol (mg%)	168 ± 7	158 ± 9	133 ± 8	144 ± 8	179 ± 9	183 ± 15
Bilirubin (mg%)	1.14 ± 0.06	0.89 ± 0.07	0.62 ± 0.04	1.06 ± 0.14	1.03 ± 0.09	1.13 ± 0.14
BUN (mg%)	18.7 ± 0.7	15.5 ± 0.7	14.5 ± 0.6	17.3 ± 3.2	15.2 ± 0.4	14.0 ± 0.9
LDH (IU/L)	678 ± 31	560 ± 38	593 ± 40	592 ± 46	642 ± 40	633 ± 55
SGPT (IU/L)	48.7 ± 5.3	39.4 ± 3.4	28.0 ± 2.7	32.8 ± 2.9	36.4 ± 4.3	32.4 ± 1.7
SGOT (IU/L)	82.6 ± 5.7	71.5 ± 4.9	74.1 ± 5.1	77.8 ± 11.3	68.9 ± 7.1	74.8 ± 6.9
ALP (IU/L)	18.9 ± 2.0	15.9 ± 1.9	14.4 ± 1.7	22.8 ± 2.0	27.4 ± 2.8*	25.2 ± 2.9*

BUN = Blood Urea Nitrogen; LDH = Lactic Dehydrogenase; SGPT = Serum Glutamic-Pyruvic Transminase; SGOT = Serum Glutamic Oxaloacetic Transaminase; ALP = Alkaline Phosphatase. Values represent the Mean ± S.E., with those differing from vehicle at $P < .05$ noted by an asterisk.

TABLE 53

Clinical Chemistry Values of Male Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Trichloroethylene (mg/ml)					
	Naive	Vehicle	0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(22)	(14)	(15)	(15)	(15)
Calcium (mg%)	10.4 ± 0.2	10.4 ± 0.2	10.5 ± 0.2	10.2 ± 0.3	11.0 ± 0.5	10.8 ± 0.3
Sodium (mEq/L)	156 ± 2	162 ± 3	160 ± 3	160 ± 2	163 ± 3	180 ± 3*
Chloride (mEq/L)	104 ± 0.5	101 ± 1	104 ± 2	104 ± 1	100 ± 2	105 ± 1
Potassium (mEq/L)	6.70 ± 0.26	7.47 ± 0.19	7.24 ± 0.15	6.42 ± 0.29*	6.79 ± 0.27	6.73 ± 0.44
Protein (g%)	7.76 ± 0.25	7.93 ± 0.17	8.21 ± 0.36	9.13 ± 0.34*	8.76 ± 0.26	7.88 ± 0.34
Glucose (mg%)	126 ± 4	114 ± 6	114 ± 7	114 ± 9	143 ± 21	129 ± 10
Cholesterol (mg%)	163 ± 7	161 ± 7	160 ± 17	155 ± 11	168 ± 11	156 ± 10
Bilirubin (mg%)	1.99 ± 0.30	1.94 ± 0.31	1.17 ± 0.07	1.36 ± 0.13	1.34 ± 0.28	0.96 ± 0.08
BUN (mg%)	20.6 ± 0.9	20.3 ± 0.5	21.0 ± 1.0	22.1 ± 1.0	23.3 ± 1.2	19.9 ± 0.9
LDH (IU/L)	893 ± 62	742 ± 40	691 ± 43	858 ± 54	841 ± 49	835 ± 48
SGPT (IU/L)	47.2 ± 5.3	37.5 ± 3.7	31.3 ± 6.7	60.7 ± 12.9	43.7 ± 3.6	45.1 ± 6.5
SGOT (IU/L)	63.0 ± 3.8	61.1 ± 4.8	61.0 ± 5.4	85.0 ± 6.5*	80.3 ± 8.0	90.4 ± 11.2
ALP (IU/L)	27.3 ± 5.6	29.9 ± 3.0	34.4 ± 8.2	32.2 ± 3.4	40.4 ± 3.2	36.7 ± 6.3

BUN = Blood Urea Nitrogen; LDH = Lactic Dehydrogenase; SGPT = Serum Glutamic-Pyruvic Transaminase; SGOT = Serum Glutamic Oxaloacetic Transaminase; ALP = Alkaline Phosphatase. Values represent the Mean ± S.E., With those differing from vehicle at $P < .05$ noted by an asterisk.

TABLE 54

Clinical Chemistry Values of Female Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(14)	(14)	(13)	(14)	(14)	(14)
Calcium (mg%)	12.4 ± 0.2	11.9 ± 0.1	13.3 ± 0.1*	13.5 ± 0.1	12.4 ± 0.1	12.4 ± 0.2
Sodium (mEq/L)	153 ± 1	155 ± 2	154 ± 1	154 ± 1	153 ± 1	153 ± 0.5
Chloride (mEq/L)	102 ± 1	100 ± 0.5	103 ± 1*	100 ± 1	100 ± 1	101 ± 1
Potassium (mEq/L)	5.99 ± 0.25	6.02 ± 0.10	5.96 ± 0.13	6.61 ± 0.30	5.91 ± 0.15	6.27 ± 0.15
Protein (g%)	7.02 ± 0.09	6.99 ± 0.01	7.31 ± 0.14	7.30 ± 0.13	7.64 ± 0.18*	7.64 ± 0.25*
Glucose (mg%)	117 ± 6	131 ± 4	133 ± 3	131 ± 4	131 ± 4	131 ± 4
Cholesterol (mg%)	102 ± 6	90 ± 6	109 ± 4	104 ± 3	117 ± 4	111 ± 5
Bilirubin (mg%)	0.82 ± 0.08	0.88 ± 0.07	0.65 ± 0.04	0.78 ± 0.03	0.99 ± 0.07	0.81 ± 0.08
BUN (mg%)	23.9 ± 0.8	31.4 ± 4.8	27.3 ± 1.2	24.7 ± 0.7	25.2 ± 0.7	25.4 ± 0.9
LDH (IU/L)	917 ± 94	723 ± 39	727 ± 45	974 ± 65	945 ± 65	910 ± 110
SGPT (IU/L)	84.6 ± 17.2	61.5 ± 6.2	43.3 ± 2.7	59.4 ± 8.5	57.9 ± 3.8	83.6 ± 20.9
SGOT (IU/L)	137 ± 12	135 ± 7	103 ± 7	110 ± 8	115 ± 6	142 ± 22
ALP (IU/L)	31.1 ± 1.6	31.7 ± 3.2	29.2 ± 2.0	33.1 ± 1.7	27.2 ± 1.7	30.1 ± 3.3

BUN = Blood Urea Nitrogen; LDH = Lactic Dehydrogenase; SGPT = Serum Glutamic-Pyruvic Transaminase; SGOT = Serum Glutamic Oxaloacetic Transaminase; ALP = Alkaline Phosphatase. Values represent the Mean ± S.E., with those differing from vehicle at $P < .05$ noted by an asterisk.

TABLE 55

Clinical Chemistry Values of Female Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(15)	(20)	(14)	(14)	(15)	(15)
Calcium (mg%)	10.8 ± 0.2	10.7 ± 0.4	10.6 ± 0.2	9.1 ± 1.5	10.5 ± 0.1	10.9 ± 0.2
Sodium (mEq/L)	150 ± 2	147 ± 2	149 ± 2	152 ± 2	152 ± 1	152 ± 1
Chloride (mEq/L)	105 ± 2	101 ± 1	108 ± 2*	108 ± 1*	110 ± 1*	105 ± 1
Potassium (mEq/L)	7.52 ± 0.59	5.67 ± 0.14	6.57 ± 0.12	6.80 ± 0.40	6.86 ± 0.35	6.34 ± 0.25
Protein (g%)	8.46 ± 0.46	8.37 ± 0.24	8.61 ± 0.39	8.02 ± 0.47	8.29 ± 0.36	7.97 ± 0.31
Glucose (mg%)	109 ± 5	118 ± 7	121 ± 6	97 ± 16	109 ± 3	127 ± 5
Cholesterol (mg%)	105 ± 8	156 ± 57	94 ± 9	187 ± 54	96 ± 5	116 ± 8
Bilirubin (mg%)	1.16 ± 0.17	1.58 ± 0.23	1.70 ± 0.29	1.52 ± 0.25	1.59 ± 0.18	1.27 ± 0.14
BUN (mg%)	26.5 ± 1.2	24.3 ± 1.3	22.9 ± 1.5	25.1 ± 1.3	22.6 ± 1.3	23.7 ± 1.1
LDH (IU/L)	736 ± 52	733 ± 34	711 ± 46	683 ± 30	701 ± 39	662 ± 39
SGPT (IU/L)	32.3 ± 3.7	52.0 ± 8.2	65.1 ± 17.2	30.2 ± 4.0	27.7 ± 3.1	30.0 ± 5.0
SGOT (IU/L)	64.3 ± 4.8	76.2 ± 4.9	91.9 ± 14.7	100.4 ± 10.2	90.0 ± 9.1	91.6 ± 8.3
ALP (IU/L)	34.0 ± 3.7	34.1 ± 4.8	30.0 ± 2.0	33.3 ± 3.8	34.8 ± 2.7	39.4 ± 4.1

BUN = Blood Urea Nitrogen; LDH = Lactic Dehydrogenase; SGPT = Serum Glutamic-Pyruvic Transaminase; SGOT = Serum Glutamic Oxaloacetic Transaminase; ALP = Alkaline Phosphatase. Values represent the Mean ± S.E., with those differing from vehicle at $P < .05$ noted by an asterisk.

TABLE 56

Hepatic Microsomal Activities in Male Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(8)	(8)	(8)	(8)	(7)	(8)
Microsomal Protein (mg/g liver)	24.6 ± .8*	28.2 ± 1.1	25.9 ± 7	25.0 ± .5*	27.0 ± .8	27.4 ± 1.0
Cytochrome P-450 (nmol/mg protein)	1.08 ± .06	1.22 ± .04	1.26 ± .11	1.25 ± .08	1.52 ± .19	1.36 ± .14
Cytochrome b_5 (nmol/mg protein)	0.410 ± .010	0.425 ± .010	0.430 ± .013	0.439 ± .013	0.477 ± .016*	0.453 ± .019
Glutathione (µmol/g liver)	5.27 ± .39	5.58 ± .21	5.85 ± .24	6.94 ± .18	7.31 ± .23	7.04 ± .20
Aminopyrine N-demethylase (nmol/mg/min)	9.30 ± .43	8.91 ± .35	10.10 ± .14*	9.79 ± .25	9.79 ± .38	9.07 ± .27
Aniline Hydroxylase (nmol/mg/min)	1.49 ± .07	1.53 ± .03	1.55 ± .05	1.52 ± .05	1.52 ± .07	1.44 ± .05

Values are given as the Mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 57

Hepatic Microsomal Activities in Male Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(7)	(8)	(8)	(8)	(8)	(8)
Microsomal Protein (mg/g liver)	32.2 ± 2.0	33.2 ± 1.5	31.9 ± 2.3	32.3 ± 1.6	30.1 ± 1.0	33.1 ± 1.0
Cytochrome P-450 (nmol/mg protein)	0.935 ± .031	0.969 ± .028	0.861 ± .022*	1.045 ± .044	0.941 ± .016	0.982 ± .051
Cytochrome b_5 (nmol/mg protein)	0.390 ± .016	0.434 ± .007	0.419 ± .018	0.470 ± .036	0.472 ± .020	0.505 ± .024*
Glutathione (μmol/g liver)	5.61 ± 0.26	4.99 ± 0.17	5.23 ± 0.21	5.40 ± 0.27	6.20 ± 0.43	5.72 ± 0.31
Aminopyrine N-demethylase (nmol/mg/min)	8.94 ± 0.48	9.80 ± 0.54	9.72 ± 0.50	11.70 ± 1.26	9.76 ± 0.54	10.24 ± 0.36
Aniline Hydroxylase (nmol/mg/min)	1.02 ± 0.03*	1.19 ± 0.02	1.14 ± 0.04	1.36 ± 0.14	1.18 ± 0.14	1.32 ± 0.07

Values are given as the Mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 58

Hepatic Microsomal Activities in Female Mice Exposed to Trichloroethylene in the Drinking Water for Four Months

Parameter	Naive	Vehicle	Trichloroethylene (mg/ml) 0.1	1.0	2.5	5.0
(Number of Mice)	(7)	(7)	(7)	(8)	(7)	(8)
Microsomal Protein (mg/g liver)	25.0 ± 1.0*	27.6 ± 0.7	28.6 ± 1.2	2.47 ± 1.1	29.6 ± 0.8	28.6 ± 1.6
Cytochrome P-450 (nmol/mg protein)	0.96 ± 0.2*	0.83 ± .04	1.05 ± 0.12*	1.09 ± .04*	1.03 ± .03*	1.03 ± .04*
Cytochrome b_5 (nmol/mg protein)	0.462 ± .014	0.457 ± .010	0.449 ± .015	0.554 ± .038*	0.497 ± .023	0.504 ± .017
Glutathione (μmol/g liver)	3.82 ± .23*	4.55 ± .08	4.41 ± .11	4.71 ± .16	4.68 ± .22	5.19 ± .41
Aminopyrine N-demethylase (nmol/mg/min)	9.72 ± .27	9.88 ± .27	9.30 ± .30	11.44 ± .37	9.81 ± .31	10.20 ± .32
Aniline Hydroxylase (nmol/mg/min)	1.32 ± .03	1.35 ± .07	1.37 ± .03	1.62 ± .06*	1.48 ± .06*	1.47 ± .11

Values are given as the Mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

TABLE 59

Hepatic Microsomal Activities in Female Mice Exposed to Trichloroethylene in the Drinking Water for Six Months

Parameter	Trichloroethylene (mg/ml)					
	Naive	Vehicle	0.1	1.0	2.5	5.0
(Number of Mice)	(8)	(8)	(8)	(8)	(8)	(8)
Microsomal Protein (mg/g liver)	27.14 ± 1.43	28.64 ± 1.29	28.12 ± 0.42	28.56 ± 0.83	30.78 ± 1.32	30.49 ± 0.75
Cytochrome P-450 (nmol/mg protein)	0.799 ± .060	0.875 ± .056	0.916 ± .064	0.769 ± .032	0.784 ± .048	0.722 ± .027*
Cytochrome b_5 (nmol/mg protein)	0.526 ± .026	0.530 ± .039	0.568 ± .030	0.528 ± .019	0.545 ± .035	0.513 ± .011
Glutathione (μmol/g liver)	4.92 ± 0.27	5.51 ± 0.23	5.22 ± 0.20	5.88 ± 0.10	5.82 ± 0.11	5.61 ± 0.14
Aminopyrine N-demethylase (nmol/mg/min)	11.08 ± 0.60	10.46 ± 0.54	10.62 ± 0.34	10.42 ± 0.44	9.66 ± 0.58	9.28 ± 0.26
Aniline Hydroxylase (nmol/mg/min)	1.56 ± 0.07	1.48 ± 0.07	1.36 ± 0.08	1.38 ± 0.08	1.31 ± 0.07	1.32 ± 0.07

Values are given as the Mean ± S.E., and those which differ significantly from the vehicle (1% emulphor) at $P < .05$ are noted by an asterisk. The doses of trichloroethylene consumed from the listed concentrations are given in Table 41.

DISCUSSION

Recent investigations in our laboratory have been aimed at incorporation of immune function assays into more standard toxicological evaluations. From the immunological viewpoint, the inbred or F_1 hybrid mouse is the animal of choice, whereas the random-bred rat would be more appropriate for a general toxicological study. As a compromise, we have chosen to use an outbred mouse, the CD-1, for our studies. Although a large number of animals per group is needed due to the variability of the immune response, standard toxicological parameters are relatively uniform. Our subchronic studies have utilized both sexes, as should be done in a comprehensive evaluation of any chemical.

It was the intent of this work to establish a data base of reference toxicology on TCE via gastrointestinal exposure. The acute toxicity of TCE appears to involve CNS depression. Since the LD_{50} was similar in both sexes, a subchronic 14-day study was performed in males to look for cumulative types of toxicity and provide data for the design of the drinking water studies. Two effects were observed in the 14-day study that are attributable to TCE. First, a 33% increase in liver weight was noted in the high dose group; secondly, a decrease in the hematocrit value was observed. The lowering of the hematocrit is congruent with a decrease (although not significant at $P < .05$) in the hemoglobin level.

Since TCE is considered as a model hepatotoxin in the rat (Pessayre et al., 1979), we considered evaluation of hepatic microsomal enzymes to be an area of importance. In an attempt to determine if alterations of liver microsomal activities could be produced, a dose of TCE equivalent to the LD_{50} was administered by daily gavage for five days. The observed alterations, consisting of increases in microsomal protein and aniline hydroxylase, and a decrease in aminopyrine N-demethylase could be attributed to starvation (Kato, 1977). Furthermore, the requirement of such a high dose is consistent with the observation by Gehring (1968) that in the female Swiss-Webster mouse, the ED_{50} of TCE to produce increased SGPT levels was 2358 mg/kg, which is near the LD_{50}.

In light of the low toxicity observed in the 14-day study with doses of 24 and 240 mg/kg, the drinking water study was set up with concentrations of TCE that would deliver comparable doses, plus two additional higher concentrations. The use of emulphor as a vehicle for TCE proved effective. Although very little effect in terms of reference toxicology was produced by these doses of TCE after either four or six month's exposure, the liver enlargement observed previously was observed. Males had increases up to 20% at four and six months; females had increases of 23% at four months but had no significant increase at six months. This apparent lack of effect at six months could be due to a high value in the vehicle group, since liver weights in the naive group were significantly lower than vehicle weights only in this set of data. Hepatic microsomal enzyme activities were largely unaltered.

Kidney weight appeared to be increased also, as a result of TCE exposure, particularly in the females. The increased liver and kidney weights observed could be expected, since the liver and kidney represent the sites of metabolism and metabolite excretion of TCE, respectively (Defalque, 1961). The fact that the high dose animals did not gain as much weight as did the other group could well be explained by the decreased fluid consumption observed in animals maintained on TCE at 5 mg/ml in the drinking water.

The changes in fibrinogen levels occurred at both the four and six month periods and may reflect an effect on the liver. The naive male group at four months shows levels that are higher than vehicle and are above the historical control. There are a few significantly low leukocyte counts, particularly in females after four months of exposure, and a few microsomal enzyme alterations (increased cytochrome P-450 and aniline hydroxylase activity) occurred in this group of animals as well. As will be seen in the following paper, the immunotoxicity of TCE was especially prominent in the females exposed for four months.

C. Effects of Trichloroethylene on the Immune System

ABSTRACT

A fourteen day range-finding study using male CD1 mice exposed to trichloroethylene (TCE) by daily oral gavage revealed inhibition of both humoral and cell-mediated immunity. Therefore, a detailed evaluation of immune status was undertaken after either four or six months' exposure of male and female mice to TCE in the drinking water. The assays used for immunological assessment were interfaced with a general toxicological evaluation previously reported (Tucker et al., 1980).

The immunological parameters assessed were humoral immunity, measured by plasma hemagglutination titers and antibody producing cells in spleens of animals sensitized to sheep erythrocytes; cell-mediated immunity, measured by delayed hypersensitivity to sheep erythrocytes; lymphocyte responsiveness, measured by the response of spleen lymphocytes to B and T cell mitogens; bone marrow function, measured by DNA synthesis and colonization of stem cells; and macrophage function, which was assessed by quantifying recruitability, adherence, chemotaxis, and phagocytosis of peritoneal exudate cells.

Females were most affected by the TCE, particularly after four months' exposure. In this group, humoral immunity was inhibited only at the highest concentrations of TCE (2.5 and 5 mg/ ml), whereas cell-mediated immunity and bone marrow stem colonization were inhibited at all four concentrations of TCE (0.1, 1.0, 2.5 and 5 mg/ml). The males were relatively unaffected after both four and six months as compared to effects observed in the fourteen-day range finding study.

Running Head: Immunotoxicology of Trichloroethylene

INTRODUCTION

This paper is the second in a series which assesses the toxicology of an environmental chemical, trichloroethylene (TCE). The first report was concerned with general toxicological effects of oral, subchronic TCE exposure in the outbred CD-1 mouse (Tucker et al., 1980). Following exposure of both sexes to four different concentrations of TCE (0.1, 1.0, 2.5, and 5.0 mg/ml) in the drinking water for four to six months, complete pathological and hematological assessment was made. The most notable effect attributed to TCE exposure was an increase in liver weight of about 20% in both sexes. However, evaluation of hepatic microsomal enzyme activities revealed no effects of exposure.

Presented against this background of relatively negative general toxicological data, the present paper reports on the effects of subchronic TCE exposure on the immune system of the mouse. The parameters assessed were humoral immunity, measured by plasma hemagglutination titers and antibody producing cells in spleens of animals sensitized to sheep erythrocytes; cell-mediated immunity, measured as delayed type hypersensitivity to sheep erythrocytes; lymphocyte responsiveness, measured by the response of spleen lymphocytes to B and T cell mitogens; bone marrow function, estimated by DNA synthesis and colonization of stem cells; and macrophage function, which was assessed by quantifying recruitability, adherence, chemotaxis, and phagocytosis of peritoneal exudate cells.

Materials and Methods

Exposure of Animals

Complete details concerning housing of the CD-1 mice and their exposure to TCE are given in the preceding paper (Tucker et al., 1980). The actual doses of TCE consumed as time weighted averages from the four concentrations in the drinking water are also given in the preceding paper. In the fourteen-day gavage study, animals were sacrificed the day after the final dose. For the drinking water study, animals were maintained on the TCE until sacrificed.

Humoral Immunity

The primary IgM response to sheep erythrocytes was measured by the hemolytic plaque assay of Jerne, as modified by Cunningham and Szenberg (1968). Mice were immunized with 4×10^8 sheep erythrocytes by i.p. injection four or five days prior to sacrifice. Spleen cell suspensions were prepared in RPMI 1640 culture medium using stainless steel mesh screens and adjusted to a cell concentration of 5×10^5/ml for assay of antibody forming cells (AFC).

As a compliment to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Seven days after i.p. injection of 10^9 sheep erythrocytes, blood was collected by cardiac puncture from chloroform-anesthetized animals into 3.2% sodium citrate. After centrifugation, the plasma was heat inactivated, and serial (1:1) dilutions were made in phosphate-buffered saline. An equal volume of a 0.5% suspension of sRBC was added to each of the dilutions in a microtiter dish. The antibody titers were expressed as $\log_2$ of the reciprocal of the first dilution where there was no visible agglutination.

Delayed-Type Hypersensitivity

Cell-mediated immunity was evaluated by measuring the delayed-type hypersensitivity response to sheep erythrocytes. Sensitization was done by injecting 10^8 erythrocytes in a volume of 20 µl into the left footpad (LFP). Four days following sensitization, the mice were challenged in the same footpad with 4×10^8 sheep erythrocytes in a volume of 40 µl. Seventeen hours following the challenge, mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (80,000 cpm/0.1 ml). Two hours later, the mice were sacrificed by cervical dislocation, and both hind feet were removed and placed in tubes for gamma counting. Paranjpe and Boone (1972) have shown that ^{125}I-albumin extravasates into the extracellular space during the delayed hypersensitivity response. The right footpad (RFP) served as an unsensitized and unchallenged control for background infiltration of ^{125}I-albumin. A group of mice challenged with sheep erythrocytes to determine nonspecific swelling served as unsensitized controls. Results are expressed as a stimulation index (S.I.) which is calculated below:

$$\text{S.I.} = \frac{\text{cpm, LFP sensitized}}{\text{cpm, RFP sensitized}} - \text{x} \; \frac{\text{cpm, LFP unsensitized}}{\text{cpm, RFP unsensitized}}$$

Lymphocyte Responsiveness to Mitogens

Lymphocyte responsiveness was assessed using the B-cell mitogen, LPS, which is lipopolysaccharide from Salmonella typhosa 0901 (Difco); and the T-cell mitogen, Con A, which is the plant lectin Concanavalin A (Sigma). For mitogenicity assays, spleens were removed aseptically and pushed through a stainless steel mesh screen to provide a single-cell suspension. Cells were diluted to 5×10^6/ml in RPMI 1640 medium supplemented with 2 mM

L-glutamine and 10% heat-inactivated fetal calf serum. Microtiter plates were prepared with 50 µl of the mitogen per well, and stored at -70°C. At the time of the assay, the plates were thawed, and 100 µl of the cell suspension was added to each of six replicate wells. LPS was used in amounts of 5, 20 and 40 µg/well; Con A was used at 1, 5 and 10 ug/well.

Plates were incubated at 37°C in 10% CO_2, 95% humidity for 48 hours. The cells were then pulsed with a ^{125}I-iododeoxyuridine/fluorodeoxyuridine solution (0.2 µCi IUdR in 1 µM FUdR). After an 18-hour incubation, the cells were collected on filter discs using a Titertek cell harvester and counted in a Beckman Gamma Counter.

Bone Marrow Assay

To assess bone marrow DNA synthesis, bone marrow cells were collected from one femur in α-MEM medium, counted, and cells per femur determined (Tucker et al., 1980). The cell count was adjusted to 3 x 10^6 cells/ml and 200 µl of cells and 20 µl of ^{125}I-IUdR/FUdR solution were added to each microtiter well. At 60, 120 and 180 minutes, 6 replicate wells were harvested per animal, and the cpm IUdR incorporated determined as described above.

To enumerate the bone marrow stem cells, bone marrow cells collected from the femur were adjusted to a cell concentration of 0.5 to 1 x 10^5/ml in 1.8% methyl cellulose in α-MEM. Suspensions were plated out in volume of 2 ml/well in 6-well Linbro plates. At the end of a seven day incubation period (37°C, 95% relative humidity, 10% CO_2), colonies were counted macroscopically and colonies/10^5 bone marrow cells plated were calculated and used as the criterion for comparison between groups.

Macrophage Function

Recruitability of macrophages was assayed following an i.p. injection of 1 ml of 10% Brewer's thioglycollate five days prior to collection of peritoneal cells using Dulbecco's Minimal Essential Media culture medium. After washing, the cells collected from the peritoneum were resuspended and counted on a Coulter counter.

Adherence of the peritoneal exudate cells was also measured. Two x 10^5 cells were placed in 24-well plastic Costar dishes and allowed to incubate for 18-24 hours at 37°C in 10% CO_2, 95% humidity. The cells were washed extensively and adherent cells were scraped from the plate and counted on a Coulter counter.

The ability of the peritoneal exudate cells to phagocytize ^{51}Cr-labelled sheep erythrocytes was measured by placing 2 x 10^5 cells in 24-well plastic Costar dishes. To each well was added 10 µl of opsonized ^{51}Cr erythrocytes and the dishes were allowed to incubate for designated times of 10 to 45 minutes, after which each well was washed and scraped. Prior to scraping, each well was exposed to a 15 sec. distilled water hypotonic shock to remove adhered erythrocytes. The cells were then counted in a Beckman counter.

Chemotaxis of peritoneal exudate cells was measured in modified blind well Boyden chambers using 13-mm Sartorius membrane filters. Varying numbers of cells (1 to 2 x 10^6 cells/ml) were placed in the upper portion of the chamber, with vehicle or chemoattractant (C5a) in the lower portion. After incubation at 37°C for 4 hours, the filters were removed, rinsed in methanol, and fixed in formalin until stained. They were then placed on glass slides, stained with hematoxylin, rinsed, dipped in 0.1% NH_4OH for 1 minute, and rinsed in distilled water. After processing through 95% etha-

nol, absolute ethanol, and isopropanol, the filters were cleared in xylene, mounted on glass slides, and dried. Using a Nikon microscope interfaced with an Artec Model 980 Image Analyzer, 20 microgrid fields were counted.

Statistical Evaluation

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's T-test was performed (Dunnett, 1964). Values which differ from vehicle control at $P < .05$ are noted in the tables with an asterisk. Each of the values in the tables is given as the mean ± standard Error (S.E.) of the mean.

RESULTS

RangeFinding Study

TCE was administered to males daily for fourteen days by gavage at doses of 24 or 240 mg/kg, which are 1/100 and 1/10 of the LD_{50}. As shown in Table 60, the humoral immune response to sheep erythrocytes was inhibited in a dose-dependent fashion, although the differences are not statistically significant at the $P < .05$ level. At the high dose, AFC/10^6 cells were 65% of control, and AFC/spleen were 68% of control. The cell-mediated immune response to sheep erythrocytes was significantly inhibited in a dose-dependent manner (Table 61). At the high dose, the stimulation index was 39% of control.

Humoral Immune Response in Mice Exposed to TCE In the Drinking Water

The humoral immune response to sheep erythrocytes was measured in male and female mice following exposure to four different levels of TCE in the

TABLE 60

Humoral Immune Response to Sheep Erythrocytes in Mice Exposed to Trichloroethylene (TCE) for Fourteen Days

TCE	N	Body Weight (g)	Spleen Weight mg	Spleen Weight %B.W.	10^3 AFC/ Spleen	AFC/10^6 Cells
Vehicle	10	30.8±0.6	183±6	0.59±0.02	3.88±0.57	2613±419
24 mg/kg	11	29.6±0.5	172±9	0.58±0.04	2.92±0.44	2140±278
240 mg/kg	9	29.4±1.6	187±15	0.63±0.03	2.63±0.58	1707±301

Trichloroethylene was administered to male CD-1 mice by daily gavage for 14 days. The vehicle was 10% emulphor in deionized water. The mice were immunized with sheep erythrocytes on day 11 and sacrificed on day 15 as described in Methods. The values represent the mean ± S.E. Antibody forming cells (AFC) per 10^6 nucleated cells and AFC per spleen are given. Values which differ from control at $P < .05$ are noted with an asterisk in all tables.

TABLE 61

Cell-mediated Immune Response to Sheep Erythrocytes in Mice Exposed to Trichloroethylene (TCE) For Fourteen Days

TCE	N	Stimulation Index
Vehicle	12	4.18 ± 0.30
24 mg/kg	12	3.13 ± 0.41*
240 mg/kg	12	1.63 ± 0.25*

CD-1 male mice, exposed to TCE as described in Table 1, were sensitized to sheep erythrocytes on day 10 and challenged on day 14. Extravasation of ^{125}I-human serum albumin was measured on day 15 and the stimulation index calculated as described in Methods. The values represent the mean $\pm$ S.E. and those which differ from control at $P<.05$ are noted with an asterisk. The value for unsensitized controls (ratio of left/right footpad) was 2.44 ± 0.26.

drinking water for four (Table 62) or six (Table 63) months. The response was inhibited after four months' exposure only in females. On the peak day of response, inhibition was n ted at the two highest doses, whether expressed as AFC/spleen or AFC/10^6 cells. The inhibition does not appear to be dose related and was no longer evident after six months' exposure to the two highest doses of TCE. There was also a significant inhibition in the males at the lowest dose after both exposure times, when enumerated on Day 4 after immunization and expressed as AFC/10^6 cells.

The hemagglutination titers to sheep erythrocytes are shown in Table 64. The inhibition observed in females after four months' exposure was apparent at the two highest doses. No inhibition was observed in males at either time or in females after six months' exposure.

Cell Mediated Immune Response in MiceExposed to TCE in the Drinking Water

The cell-mediated immune response to sheep erythrocytes was evaluated by the delayed hypersensitivity assay. As shown in Table 65, females exposed to TCE for four months had a depressed stimulation index at all four doses, whereas only at the high dose after six months was the stimulation index depressed. There was no effect in males at either exposure time.

Mitogenicity

The response of spleen lymphocytes to the T-cell mitogen, Con A, and the B-cell mitogen, LPS, was determined in the animals exposed to TCE in the drinking water for six months. The background response in medium without mitogen is shown in Table 66 and appears to increase as a result of TCE exposure, although only one value is significantly different from control. This background is not subtracted from the mitogen-treated cul-

TABLE 62

Humoral Immune Response to Sheep Erythrocytes in Mice Exposed to Trichloroethylene (TCE) for Four Months

	Day 4		Day 5	
TCE	10^3 AFC/spleen	AFC/10^6	10^3 AFC/spleen	AFC/10^6 cells
I. Males				
Naive	2.66±0.27	1534±159	1.11±0.11	577±99
Vehicle	2.53±0.24	1832±306	1.16±0.11	773±161
0.1 mg/ml	1.74±0.28	791±183*	1.39±0.31	508±93
1.0 mg/ml	2.30±0.23	2063±224	1.16±0.15	637±96
2.5 mg/ml	2.37±0.22	1622±274	0.92±0.18	671±143
5.0 mg/ml	2.56±0.32	1921±312	0.56±0.07	599±65
II. Females				
Naive	2.27±0.35	1426±173	0.46±0.09	206±54
Vehicle	1.93±0.08	2038±154	0.55±0.08	272±67
0.1 mg/ml	1.36±0.07*	1718±231	0.50±0.04	313±36
1.0 mg/ml	1.97±0.18	1289±335	0.57±0.05	323±56
2.5 mg/ml	1.23±0.15*	639±100*	0.51±0.05	257±40
5.0 mg/ml	1.36±0.21*	732±129*	0.41±0.04	246±17

Trichloroethylene was administered in the drinking water to male and female CD-1 mice as described in Methods. The naive group received deionized water and the vehicle control group received 1% emulphor. Immunization and enumeration of antibody forming cells (AFC) was done as in [1]. N is 12 for vehicle, 7 for all other groups. Values which differ from vehicle control at $P \leq .05$ are noted with an asterisk.

TABLE 63

Humoral Immune Response to Sheep Erythrocytes in Mice Exposed to Trichloroethylene (TCE) for Six Months

TCE	Day 4		Day 5	
	10^3 AFC/spleen	AFC/10^6 cells	10^3 AFC/spleen	AFC/10^6 cells
I. Males				
Naive	1.39±0.21	1744±116	3.26±0.63	2643±362
Vehicle	2.08±0.26	1813±178	3.49±0.52	2936±235
0.1 mg/ml	1.32±0.37	890±132*	2.58±0.56	2572±443
1.0 mg/ml	1.51±0.26	1498±283	3.17±0.52	2997±513
2.5 mg/ml	2.09±0.32	1612±203	1.79±0.44*	1913±412
5.0 mg/ml	1.42±0.27	1186±213*	1.84±0.32*	1772±293
II. Females				
Naive	1.99±0.45	2103±506	1.99±0.16	1958±160
Vehicle	2.28±0.33	1952±274	1.64±0.34	1515±248
0.1 mg/ml	1.53±0.46	1209±353	1.44±0.20	1433±174
1.0 mg/ml	2.29±0.59	1680±347	1.14±0.26	1128±223
2.5 mg/ml	1.52±0.29	1304±209	1.02±0.13	978±150
5.0 mg/ml	2.17±0.53	1661±380	1.12±0.18	1158±206

Trichloroethylene was administered in the drinking water to male and female CD-1 mice as described in Methods. The naive group received deionized water and the vehicle control group received 1% emulphor. Immunization and enumeration of antibody forming cells (AFC) was done as in . N is 12 for vehicle, 7 for all other groups. Values which differ from vehicle control at $P<.05$ are noted with an asterisk.

TABLE 64

Hemagglutination Titers in Mice
Exposed to Trichloroethylene (TCE) for
Four or Six Months

		Males		Females	
I.	TCE	N	Log 2 Titer	N	Log 2 Titer
Four Months	Naive	14	9.18±.29	14	10.61±.29
	Vehicle	25	8.84±.13	25	9.96±.25
	0.1mg/ml	15	8.59±.12	15	10.26±.30
	1.0mg/ml	15	8.72±.19	15	9.46±.24
	2.5mg/ml	15	8.68±.13	14	9.11±.21*
	5.0mg/ml	15	9.12±.11	15	8.59±.18*
II.					
Six Months	Naive	14	8.75±.17	14	8.25±.16
	Vehicle	23	8.59±.22	23	8.58±.13
	0.1mg/ml	15	8.92±.25	15	8.72±.16
	1.0mg/ml	15	9.59±.36*	15	8.06±.18
	2.5mg/ml	15	9.19±.29	15	8.25±.24
	5.0mg/ml	18	8.27±.19	15	8.46±.19

Hemagglutination titers to sheep erythrocytes were determined as described in Methods and are expressed as $\log_2$ of the reciprocal of the dilution shown not to agglutinate. Values are mean ±S.E. and those which differ from vehicle control at $P<.05$ are noted with an asterisk.

TABLE 65

Cell-mediated Immune Response to Sheep Erythrocytes in Mice Exposed to Trichloroethylene (TCE) for Four or Six Months

		Males		Females	
I.	TCE	N	SI	N	SI
Four Months	Naive	15	2.85±.36	15	4.54±.59
	Vehicle	19	4.04±.40	20	5.41±.43
	0.1 mg/ml	15	2.99±.34	15	3.00±.50*
	1.0 mg/ml	15	3.16±.30	15	3.82±.60*
	2.5 mg/ml	14	4.83±.70	15	2.60±.32*
	5.0 mg/ml	15	4.13±.58	15	2.86±.20*
II.					
Six Months	Naive	12	3.42±.61	13	3.71±.45
	Vehicle	19	4.32±.28	18	4.49±.33
	0.1 mg/ml	12	3.92±.33	15	3.76±.35
	1.0 mg/ml	14	3.17±.33	14	3.68±.27
	2.5 mg/ml	14	3.90±.35	13	3.30±.41
	5.0 mg/ml	20	4.43±.42	17	1.38±.53*

Trichloroethylene was administered in the drinking water as described in Methods. The naive group received deionized water and the vehicle group 1% emulphor. The Stimulation Index (SI) was calculated as in . Values for unsensitized controls were 2.10 for 4 month males, 1.61 for 6 month males, 2.50 for 4 month females, and 1.67 for 6 month females.

TABLE 66

Lymphocyte Responsiveness in the Absence of Mitogen Using Spleens from Mice Exposed to Trichloroethylene (TCE) for Six Months

	Males		Females	
TCE	N	IUDR (cpm)	N	IUDR (cpm)
Naive	15	7440±1173	15	5715±552
Vehicle	23	4856±1147	23	8045±812
0.1 mg/ml	15	3662±496	15	8025±641
1.0 mg/ml	15	6137±808	15	5227±360
2.5 mg/ml	15	6120±716	15	11820±2403
5.0 mg/ml	20	10516±1084*	15	10722±1692

Spleen lymphocyte responsiveness was estimated from uptake of ^{125}I-IUDR as described in Methods. The values represent the mean cpm ±S.E. for the number of mice indicated, each mouse having six replicate measurements. Values which differ from vehicle control at $P<.05$ are noted by an asterisk.

tures. Data on the mitogen response for males is given in Table 67 that for females in Table 68. In neither sex was there an effect on splenic lymphocyte responsiveness to these two mitogens.

Assessment of Bone Marrow Function

The ability of bone marrow stem cells to form colonies in semi-solid medium was used as one parameter for assessment of bone marrow function in mice receiving TCE in the drinking water (Table 69).

In females, the ability of stem cells to colonize was significantly inhibited at all doses at both the four-and six-month exposure times. In males, although the values at four months are significantly decreased in the treated groups, this may be due to a high vehicle value, since the naive value is also significantly lower than the vehicle. After six months' exposure, the values from the three highest treatment groups are in fact higher than vehicle control.

DNA synthesis, estimated by uptake of ^{125}I-iododeoxyuridine, was used as another assessment of bone marrow function. This data is shown in Table 70. The only effect which appears to be related to treatment is a slight stimulation of uptake observed in females after four months' exposure.

Macrophage Function

Specific macrophage functions were assessed in mice exposed to TCE in the drinking water. Data on recruitability of peritoneal exudate cells is shown in Table 71 The number of cells recruited by thioglycollate was reduced in males exposed for four months but not six months. Recruitment was generally unaffected in females, although two values are higher than the control in the 6 month animals. Adherence of recruited cells (Table 72) was markedly depressed in males after four months' exposure, although

TABLE 67

Lymphocyte Responsiveness to Concanavalin A (Con A) and Bacterial Lipopolysaccharide (LPS) Using Spleens from Male Mice Exposed to Trichloroethylene (TCE) for Six Months

TCE	N	Con A (1ug)	N	LPS (5ug)
Naive	15	105059±4006	15	77284±4885
Vehicle	23	84278±7559	23	71081±6031
0.1 mg/ml	15	77005±13025	15	64363±10304
1.0 mg/ml	15	90917±8158	15	86542±5344
2.5 mg/ml	15	80070±7217	15	75359±5106
5.0 mg/ml	20	107903±5279	20	80500±4473
TCE	N	Con A (5ug)	N	LPS (20ug)
Naive	15	120359±4701	15	67240±4945
Vehicle	23	117061±7506	23	66763±6302
0.1 mg/ml	15	97073±16103	15	58807±9563
1.0 mg/ml	15	132190±6050	15	81065±4219
2.5 mg/ml	15	107149±9189	15	74423±5631
5.0 mg/ml	20	126796±4125	20	77584±4474
TCE	N	Con A (10ug)	N	LPS (40ug)
Naive	15	110116±6390	15	62242±4534
Vehicle	23	87742±8748	23	61680±6249
0.1 mg/ml	15	81503±15667	15	57854±9472
1.0 mg/ml	15	126775±9673	15	78808±5066
2.5 mg/ml	15	91616±10708	15	65322±5899
5.0 mg/ml	20	115077±7934	20	73971±5657

The values represent the mean ± S.E. calculated as follows: $\frac{\text{total cpm}}{6} \times \frac{\text{cpm on day 0}}{\text{cpm on day of assay}}$. Calculation enclosed in parenthesis corrects for decay over counting period.

TABLE 68

Lymphocyte Responsiveness to Concanavalin A (Con A) and Bacterial Lipopolysaccharide (LPS) Using Spleens from Female Mice Exposed to Trichloroethylene (TCE) for Six Months

TCE	N	Con A (1 µg)	N	LPS (5 µg)
Naive	15	61139 ±8484	15	42476 ±5734
Vehicle	23	69459 ±7586	23	44711±3696
0.1 mg/ml	15	75171 ±9084	15	48566±4690
1.0 mg/ml	15	68148 ±4972	15	40882 ±3327
2.5 mg/ml	15	61136 ±7710	15	35507±4677
5.0 mg/ml	15	80315 ±7439	15	47672±4416
TCE	N	Con A (5 µg)	N	LPS (20 µg)
Naive	15	106262 ±9291	15	42673±5631
Vehicle	23	99649 ±6921	23	46801±2980
0.1 mg/ml	15	80760 ±8851	15	46491±4340
1.0 mg/ml	15	110674 ±5951	15	37750±3545
2.5 mg/ml	15	65550 ±8487	15	39332±5294
5.0 mg/ml	15	95520 ±7461	15	48975±4471
TCE	N	Con A (10 µg)	N	LPS (40 µg)
Naive	15	74611 ±9967	15	44750±5911
Vehicle	23	83262 ±8382	23	47953±2929
0.1 mg/ml	15	81252 ±9577	15	49043±4731
1.0 mg/ml	15	83060 ±6727	15	38722±3567
2.5 mg/ml	15	61554 ±9550	15	37268±5296
5.0 mg/ml	15	90586 ±9657	15	49727±4581

The values represent the Mean ± S.E., calculated as described in Table 67.

TABLE 69

Bone Marrow Stem Cells in Mice Exposed to Trichloroethylene (TCE) for Four or Six Months

		Males		Females	
I.	TCE	N	Colonies	N	Colonies
Four Months	Naive	12	159±8*	14	189±7
	Vehicle	25	215±3	26	213±5
	0.1 mg/ml	14	183±7*	13	176±5*
	1.0 mg/ml	15	189±6*	12	159±4*
	2.5 mg/ml	14	178±5*	14	154±5*
	5.0 mg/ml	15	184±4*	15	140±6*
II.					
Six Months	Naive	13	165±2	15	222±3
	Vehicle	23	170±2	23	229±2
	0.1 mg/ml	14	177±2	14	206±2*
	1.0 mg/ml	15	196±2*	14	194±1*
	2.5 mg/ml	14	197±2*	11	178±3*
	5.0 mg/ml	20	204±1*	13	169±2*

The formation of colonies was used to enumerate bone marrow stem cells as described in Methods. Values are colonies/10^5 cells and are expressed as the mean ± S.E., those differing at $P<.05$ being noted by an asterisk.

TABLE 70

Bone Marrow DNA Synthesis in Mice Exposed to Trichloroethylene (TCE) for Four or Six Months

I. Four Months

	Males			Females		
TCE	60 Min	120 Min	180 Min	60 Min	120 Min	180 Min
Naive	821±117	2469±368	3141±377	1061±162	2495±250*	3360±236
Vehicle	907±70	2447±209	3252±220	908±86	1822±180	3061±172
0.1 mg/ml	828±103	2030±180	2810±259	1031±142	1930±276	2547±236
1.0 mg/ml	1019±135	2561±300	3161±303	1444±139	2698±167*	3049±235
2.5 mg/ml	1002±229	2708±498	4261±280	2199±271*	3345±344*	2584±275
5.0 mg/ml	1186±276	3298±418	4227±440	1901±202*	3292±330*	3348±190

II. Six Months

	Males			Females		
TCE	60 Min	120 Min	180 Min	60 Min	120 Min	180 Min
Naive	938±87	1643±169	2115±197	1158±104	2192±169	2673±140
Vehicle	1170±106	1946±146	2352±133	1395±109	2733±209	3213±162
0.1 mg/ml	966±85	1999±145	2434±151	1135±106	2326±106	2987±157
1.0 mg/ml	826±96	1704±120	2291±138	1303±151	1879±189*	2546±221
2.5 mg/ml	1031±138	1891±197	2554±216	1724±202	2970±307	2980±226
5.0 mg/ml	1155±100	2390±170*	2580±127	1244±174	2475±281	2586±239

DNA synthesis was estimated from incorporation of ^{125}I-iododeoxyuridine as described in Methods. The values in the table are given as Mean cpm ± S.E. from six replicate samples of each animal, where the number of animals per group was 25 for vehicle and 15 for all other groups. Uptake was determined after 60, 120 and 180 minutes. Values which differ from vehicle control at $P < .05$ are noted by an asterisk.

TABLE 71

Recruitment of Peritoneal Exudate Cells in Mice Exposed to Trichloroethylene (TCE) for Four or Six Months

	Males		Females	
TCE	4 Months	6 Months	4 Months	6 Months
Naive	38 ± 7	14 ± 3	26 ± 3	15 ± 2
Vehicle	50 ± 8	18 ± 4	18 ± 4	20 ± 2
0.1 mg/ml	29 ± 4*	19 ± 5	24 ± 4	18 ± 1
1.0 mg/ml	26 ± 4*	19 ± 2	17 ± 2	21 ± 3
2.5 mg/ml	18 ± 3*	21 ± 4	23 ± 4	25 ± 2*
5.0 mg/ml	22 ± 2*	21 ± 4	26 ± 7	26 ± 1*

Cells were recruited by injection of thioglycollate seven days prior to sacrifice as described in Methods. Values are 10^6 cells/mouse and are expressed as Mean ± S.E., (N=10), and those which differ from control at $P < .05$ are noted with an asterisk.

TABLE 72

Adherence of Peritoneal Exudate Cells
in Mice Exposed to Trichloroethylene (TCE)
for Four or Six Months

	Males		Females	
TCE	4 Months	6 Months	4 Months	6 Months
Naive	18.6±5.6	5.2±0.9	14.0±2.8	4.5±0.3
Vehicle	19.5±7.0	7.6±1.8	9.6±2.5	4.8±0.8
0.1 mg/ml	11.0±3.9	not done	14.8±1.8	7.9±0.7*
1.0 mg/ml	6.1±0.5*	not done	14.0±2.5	6.5±1.0
2.5 mg/ml	3.8±0.3*	3.6±0.5	8.6±0.9	9.6±1.2*
5.0 mg/ml	4.3±0.5*	5.0±1.4	6.4±1.0	9.9±1.3*

Adherence of thioglycollate recruited cells was measured as described in Methods. The values are 10^5 cells and are expressed as the mean ± S.E. (N=5), and those which differ from vehicle at $P<.05$ are noted with an asterisk.

the degree of depression after six months was not evident. Again, a slight increase was seen in the females after six months' exposure. There were no consistent dose-dependent changes in chemotaxis (Table 73). The data on phagocytosis of sheep erythrocytes by macrophages recruited from mice exposed to TCE for 4 months is shown in Figure 32 (males) and Figure 33 (females). There was a stimulation at one dose in males and at two doses in females. There were no differences observed after six months' exposure (data not shown).

Discussion

The toxicological effects (other than immune) of subchronic 14-day exposure to TCE by the oral route were generally negative, slight hepatomegaly being the primary effect (Tucker et al., 1980). However, in the 14-day range finding study using male mice, both humoral and cell mediated immunity were inhibited in a dosedependent fashion (Tables 60 - 61). A more detailed evaluation of the immune status was therefore conducted on mice exposed to TCE in the drinking water for four and six months. In these studies, it is important to note that sensitization to the antigen, sheep erythrocytes, took place during the TCE exposure period.

The humoral immune response, as measured by enumeration of spleen antibody forming cells and hemagglutination titers, was inhibited in female mice; however, this suppression was no longer evident after six months' exposure. Possible peak suppression occurred earlier in the exposure period with some form of tolerance occurring by the sixth month. The males showed a slight inhibition of antibody forming cells/spleen after 6 months exposure. However, this suppression was not demonstrated with the hemagglutination titers. It should be noted that the baseline hemagglutination

TABLE 73

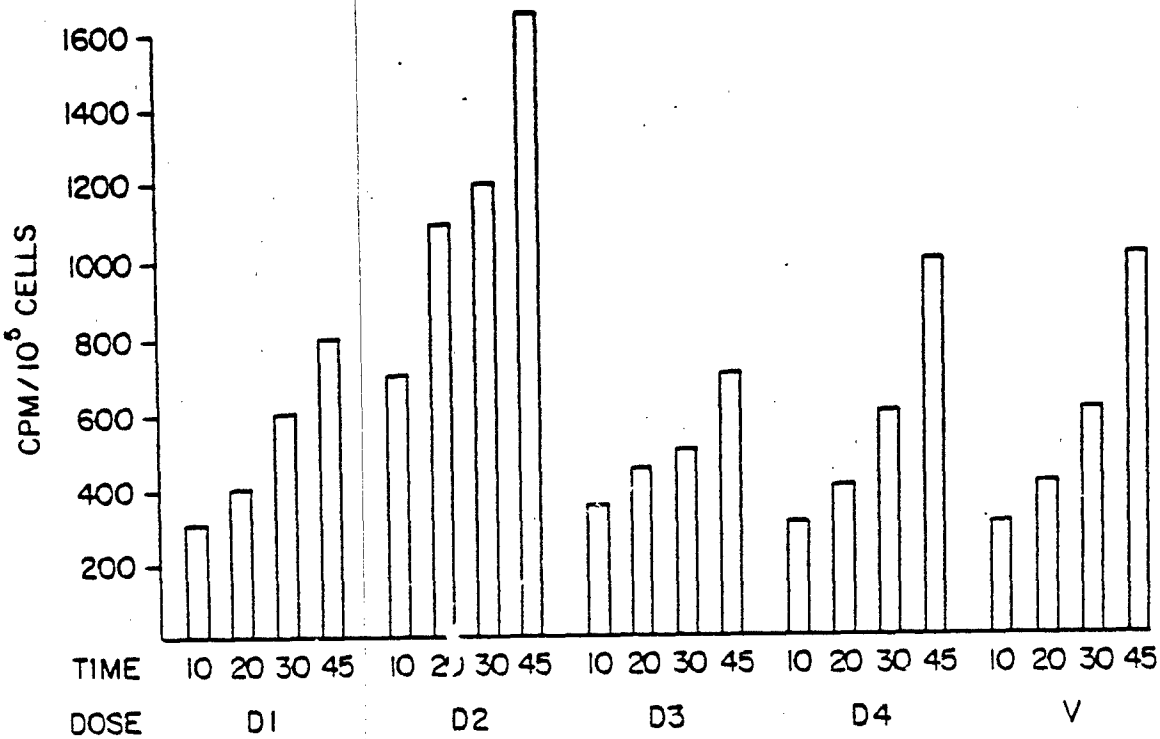
Chemotaxis of Recruited Peritoneal Exudate Cells in Mice Exposed to Trichloroethylene (TCE) for Four or Six Months

I. Males	4 Months		6 Months	
TCE	Control	+C5a	Control	+C5a
Naive	8.4±2.9	20.3±3.2	2.1±0.6	29.7±3.0
Vehicle	9.5±2.3	15.0±1.8	3.0±0.7	42.0±5.5
0.1 mg/ml	7.5±3.0	24.1±5.3	3.5±0.9	20.0±4.9
1.0 mg/ml	7.7±3.9	29.2±2.2	4.0±0.9	41.0±0.8 *
2.5 mg/ml	13.0±4.3	58.3±7.8*	4.7±1.3	20.3±2.3
5.0 mg/ml	not done	not done	8.3±1.6*	27.4±4.9

II. Females	4 Months		6 Months	
TCE	Control	+C5a	Control	+C5a
Naive	5.0±0.8	12.8±1.7	8.3±1.0	38.8±8.3
Vehicle	7.1±1.4	19.0±2.3	11.3±1.6	42.3±5.5
0.1 mg/ml	4.9±1.8	12.4±1.9	7.5±1.3	30.0±2.7
1.0 mg/ml	6.4±1.1	10.5±1.5	10.2±0.9	42.3±5.2
2.5 mg/ml	6.7±0.8	23.3±3.2*	5.5±1.0*	39.2±4.8
5.0 mg/ml	2.8±1.1	11.3±2.8	7.9±2.6	55.6±9.1

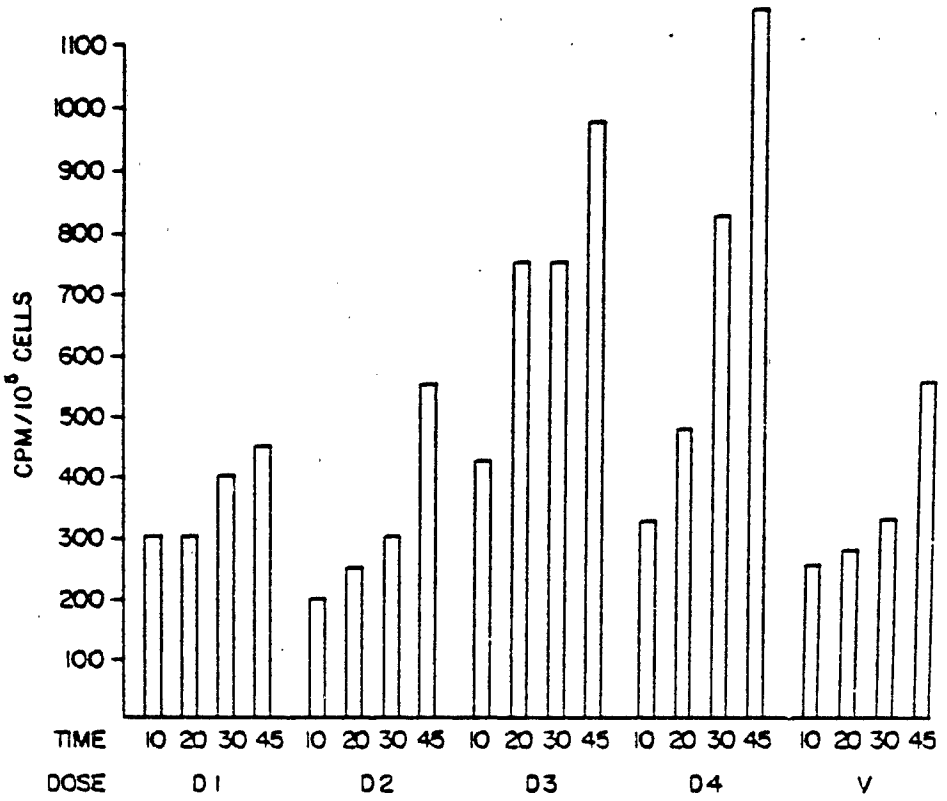
Chemotaxis was performed on thioglycollate--recruited cells as described in Methods, using Boyden chambers with and without the chemoattractant C5a. The values are number of cells per field and are expressed as the mean ± S.E. (N=10), and those which differ from vehicle at $P<.05$ are noted by an asterisk.

FIGURE 32



Phagocytosis by recruited peritoneal exudate cells from male mice exposed to trichloroethylene (TCE) for four months. Phagocytosis of opsonized ^{51}Cr-labelled sheep erythrocytes was measured as described in Methods. The bars in the figure represent cpm/10^5 cells after incubation for 10, 20, 30 or 45 minutes. The concentration of TCE is indicated as D1 for 0.1 mg/ml, D2 for 1.0 mg/ml, D3 for 2.5 mg/ml and D4 for 5.0 mg/ml. Only the vehicle control was assayed.

FIGURE 33



Phagocytosis by peritoneal exudate cells from female mice exposed to trichloroethylene (TCE) for four months. Phagocytosis of opsonized ^{51}Cr-labelled sheep erythrocytes was measured as described in Methods. The bars in the figure represent cpm/10^5 cells after incubation for 10, 20, 30 or 45 minutes. The concentration of TCE is indicated as D1 for 0.1 mg/ml, D2 for 1.0 mg/ml, D3 for 2.5 mg/ml and D4 for 5.0 mg/ml. Only the vehicle control was assayed.

titers were decreased 30% and 80% for males and females respectively between the fourth and sixth month. Lymphocyte response to the B cell mitogen, LPS, was not altered after four or six months' exposure. Thus, the humoral immune suppression to TCE was most markedly manifested in female mice after four months' exposure using spleen antibody forming cell response as the indicator.

Cell mediated immunity, measured by delayed hypersensitivity response to sheep erythrocytes, was markedly suppressed in the females after four months' exposure, but not after six months; whereas the males were unaffected at both exposure times. This absence of an effect in males is in contrast to the 67% inhibition seen in the subchronic 14-day gavage study (Table 61). Two factors must be considered in this data. First, exposure in the 14-day study was by daily gavage. Here, the bolus of TCE is administered and the kinetics of absorption, metabolism and excretion are, without a doubt, quite different from semi-continuous self administration. Secondly, the exposure time is an important variable. The subchronic four and six month exposure allows time for the induction of TCE metabolism, excretion of the chemical, and a recovery of the biological (immune) effects. There was no marked effect on the hepatic microsomal parameters (Tucker et al., 1980), but the metabolism of TCE was not studied. Lymphocyte response to the T cell mitogen, Concanavalin A, was not affected in either sex at either exposure time period. This suggests that the proliferation capacity of the T lymphocyte is intact and the possibility that the TCE induced suppression of cell mediated immunity is on the effector arm of the response. In ongoing studies, we are measuring the popliteal lymph node proliferative capacity during the delayed hypersensitivity response which should provide additional information relating to the site of action.

The bone marrow was another target for toxicity in female mice. TCE's effect on the monocyte-granulocyte progenitor cells was present after four and six months' exposure. The effect was dose dependent and, although not a remarkable effect, may have caused a reduction in the number of functions of the monocytes needed for the integrity of the immune responses. The status of the progenitor cells in males, though showing some statistical changes, are not considered of biological consequence because the vehicle value is significantly different from naive mice, the lack of dose dependence, and the small diffrerences seen.

Specific macrophage functions were generally unaffected by subchronic TCE exposure. Although the number of recruitable peritoneal exudate cells was reduced in the males after exposure for four months, the vehicle exposed group is out of line with our historical controls. Fiedler et al. showed that increased chlorination of the water decreased the number and tumoricidal activity of recruitable peritoneal exudate cells. This does not appear to be the situation with this chlorinated chemical. Of the recruited cells from male mice exposed for four months, there was a reduction in the number of cells that could adhere to plastic. There were no consistent changes in the recruited cells' ability to move either randomly or directed towards a chemotactic factor. Similarly, there were no consistent changes in the phagocytic activity of the adhered peritoneal exudate cells. The data from the peritoneal cells generally appears normal and does not support the decrease in bone marrow progenitor cells. It may be that the sensitivity of the recruitment and adherence data is not sufficient to support the modest changes seen in bone marrow progenitor cell number.

Although the effects of subchronic oral exposure to TCE on the immune system are not remarkable, even at the highest doses employed in this study, this system represents a target for this chemical. The effect of TCE on the immune system is consistent with effects of other chlorinated hydrocarbons which have been shown to affect the immune system (Loose et al., 1979). The females are especially sensitive to TCE, and suppression of certain immune parameters, particularly spleen antibody forming cells to sheep erythrocytes, is time related in that the effects are more pronounced at four rather than at six months. The males were relatively unaffected by four or six months' exposure as compared to the 14-day range finding study. This immunosuppression demonstrated by the females is interesting in light of the recent work done by Henschler et al. (1980). In these experiments the aminebase stabilized trichloroethylene promoted the development of lymphomas in random-bred female mice exposed via longterm inhalation.

The data presented in this and previous reports demonstrate the feasibility of a broad based immunotoxicological assessment of effects of oral administration of a relatively water insoluble and valatile environmental chemical using a random-bred mouse. Although this experimental animal presents variability problems in the immune assay, it provides a bridge to the random-bred rat used in most toxicological studies. Future reports will describe the effects of other environmental chemicals.

D. GENERAL TOXICOLOGY OF DEXAMETHASONE

Introduction

Dexamethasone, a gift from Merck, Sharpe and Dohme, was selected as the known immunosuppressant agent for these studies. It has been used as an anti-inflammatory agent in man for over a decade, and its immunosuppressant effects in rodents are well established.

The stability of the disodium phosphate of dexamethasone (9α-fluoro-16α-methylprednisolone) was assessed using high pressure liquid chromatography. The stability tests were performed because the subchronic 90-day exposures were in the drinking water and the solutions were changed twice weekly. The maximum loss of dexamethasone over the four-day period was 34% at the lowest concentration tested, and 8% at the highest concentration tested. The results of one of these studies are shown in Table 74.

Acute Toxicity

Acute toxicity studies were performed on CD-1 male and female mice using a single gavage. These mice survived 1 g/kg dexamethasone. We have chosen not to give higher doses because the information gained would not relate appreciably to the exposure by drinking water. Information provided by Mercke, Sharpe and Dohme indicated the LD50 to be about 600 mg/kg. We do not know how to reconcile this difference except to suggest that the mice were from different sources.

Subchronic 14-Day Exposure

Two subchronic experiments were set up whereby mice were exposed to dexamethasone in the drinking water. In the first experiment, five concentrations from 0.3 μg/ml to 10.0 μg/ml were used. Body weights were measured five times over the 2 week period and at the end of the exposure period organ weights and leukocyte counts were determined. Table 75 shows body weight data from mice weighed on days 0, 4, 8, 11 and 15. Mice dosed at 2.5 and 10.0 μg/ml showed significant weight loss toward the end of the exposure period.

Table 76 represents data collected on the organ weights from these mice. The data are expressed as percent body weight. Spleen and thymus weights were significantly decreased as compared to controls in a dose response manner; the higher the dose of dexamethasone, the smaller the spleen and thymus. Surpri-

TABLE 74

Stability of Dexamethasone Phosphate in Deionized Water

Sample Number	DAY 0		DAY 1 (24 hr)		DAY 2 (48 hr)		DAY 3 (72 hr)		DAY 4 (96 hr)	
	Conc (µg)	Peak Height (cm)	Conc (µg)	Peak Height (cm)	Conc (µg)	Peak Height (cm)	Conc (µg)	Peak Height (cm)	Conc (µg)	Peak Height (cm)
1	0.29	4.25	0.25	3.70	0.23	3.39	0.22	3.15	0.19	2.92
2	0.52	7.30	0.47	6.70	0.44	6.38	0.44	6.37	0.42	6.09
3	0.71	10.00	0.69	9.45	0.64	9.04	0.63	8.97	0.60	8.58
4	0.91	12.80	0.84	11.80	0.82	11.64	0.82	11.64	0.79	11.15
5	1.23	16.90	1.14	16.00	1.10	15.60	1.10	15.60	1.06	14.90
6	1.29	18.10	1.24	17.40	1.24	17.40	1.23	17.30	1.20	16.90
7	1.50	20.00	1.42	19.90	1.43	20.00	1.40	19.70	1.38	19.40

TABLE 75

Body Weight (in grams) of Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Fourteen Days

Group	DAYS 0	4	8	11	15
Vehicle	26.4 ± 0.7	28.2 ± 0.6	27.9 ± 0.6	28.8 ± 0.6	28.7 ± 0.5
0.3 µg/ml	27.4 ± 0.4	27.9 ± 0.6	28.7 ± 0.6	28.7 ± 0.5	27.9 ± 0.5
0.6 µg/ml	27.8 ± 0.5	28.9 ± 0.6	28.9 ± 0.6	29.1 ± 0.4	28.7 ± 0.4
1.25 µg/ml	28.0 ± 0.5	28.2 ± 0.5	27.8 ± 0.4	27.8 ± 0.4	27.2 ± 0.4
2.50 µg/ml	27.4 ± 1.1	27.2 ± 0.9	25.9 ± 1.0	26.0 ± 0.9	24.9 ± 1.0*
10.00 µg/ml	27.9 ± 0.5	27.3 ± 0.6	25.5 ± 0.6*	25.4 ± 0.6*	23.0 ± 0.8*

Values (in grams) represent the mean ± SE derived from 5 mice per group. Those values which differ significantly from the vehicle control at $p < 0.05$ are noted by an asterisk.

TABLE 76

Organ Weights as Percent of Body Weight and Leukocyte Counts of Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Fourteen Days

Group	Spleen (% body weight)	Thymus (% body weight)	Leukocytes (x $10^3/mm^3$)
Vehicle	0.54 ± 0.02	0.26 ± 0.02	10.8 ± 1.3
1.3 µg/ml	0.56 ± 0.04	0.25 ± 0.02	12.7 ± 1.0
0.6 µg/ml	0.48 ± 0.02	0.22 ± 0.02	13.0 ± 0.8
1.25 µg/ml	0.39 ± 0.02*	0.19 ± 0.01*	9.8 ± 0.8
2.50 µg/ml	0.30 ± 0.02*	0.10 ± 0.01*	8.3 ± 1.0
10.00 µg/ml	0.19 ± 0.02*	0.10 ± 0.01*	7.7 ± 1.3

Values represent the mean ± SE derived from 5 mice per group. Those values which differ significantly from vehicle control at $p < 0.05$ are noted by an asterisk.

singly, no significant changes were observed in leukocyte counts.

A second study was performed using concentrations between 1.25 µg/ml and 0.1 µg/ml. Table 77 summarizes the data. The mice receiving 1.25 µg/ml in the drinking water did not gain weight at the same rate as the controls (Table 75). The only dexamethasone-induced organ weight change was a reduction in spleen weight. Thymic involution did not occur in this experiment at the 1.25 µg/ml level. In the first experiment, the thymic weight was decreased 26%. Based on the body and organ weight data, the concentration in the drinking water between 1/25 µg/ml and 2.5 µg/ml should produce effects. Table 78 shows the hematologic data on mice exposed to dexamethasone. There were no effects that were statistically different from controls. If the number of mice per group had been larger, a statistically significant decrease in leukocytes might have occurred. Mice exposed to dexamethasone had a higher microsomal protein content per gram of liver (Tables 79 and 80). Mice exposed to 0.6 µg/ml and 1.25 µg/ml had a 34% and 49% increase in microsomal protein. No other parameters were altered.

Based on these two range-finding studies, the concentrations of dexamethasone in the drinking water for the 90-day study were set at 0.2 µg/ml, 1.0 µg/ml and 2.0 µg/ml. This study provides a positive control for all the in vitro studies and provides a no observable effect level and a pronounced effect level.

Subchronic 90-Day Exposure

The data on consumption of dexamethasone by the mice are shown in Table 81. Only the male mice showed a decrease in fluid consumption. Mice with 2.0 µg/ml dexamethasone in the drinking water consumed 45% and 36% less fluid when calculated as ml/mouse/day and ml/kg/day. The calculated time-averaged doses delivered based on fluid consumption and body weights over the 90-day exposure period were .04, .22 and .39 mg/kg for males and .04, .22 and .44 mg/kg for the females.

Dexamethasone produced a dose-dependent retardation of growth for both males and females. The effect on growth rate is more difficult to assess, but it appears that the plateau phase of growth occurred earlier in the dexamethasone-exposed groups. The changes in body weights over the 90-day period are shown in Table 82. The females appeared to be slightly more sensitive to the body weight changes than the males.

TABLE 77

Body and Organ Weights of Weanling Female CD-1 Mice

Exposed for Fourteen Days to Dexamethasone in Drinking Water

Treatment	Number Mice	Body Weight (g)	Organ	Weight (mg)	% Body Weight	Organ/Brain Ratio
Vehicle	5	25.8 ± .45	Brain	451.6 ± 13.0	1.75 ± 0.07	-
			Liver	1321.4 ± 83.8	5.11 ± 0.28	2.95 ± 0.26
			Spleen	191.0 ± 19.5	0.74 ± 0.07	0.42 ± 0.05
			Lungs	195.6 ± 11.7	0.76 ± 0.05	0.43 ± 0.02
			Thymus	75.6 ± 6.0	0.29 ± 0.02	0.17 ± 0.01
			Kidneys	347.8 ± 15.4	1.35 ± 0.06	0.77 ± 0.03
0.1 µg/ml	5	25.0 ± .30	Brain	476.4 ± 10.2	1.90 ± 0.04	-
			Liver	1253.0 ± 70.5	5.01 ± 0.33	2.64 ± 0.17
			Spleen	168.2 ± 17.1	0.67 ± 0.07	0.35 ± 0.03
			Lungs	209.6 ± 10.5	0.84 ± 0.05	0.44 ± 0.02
			Thymus	90.0 ± 7.7	0.36 ± 0.03	0.19 ± 0.02
			Kidneys	368.6 ± 18.5	1.47 ± 0.08	0.77 ± 0.03
0.3 µg/ml	5	25.6 ± .57	Brain	436.0 ± 6.0	1.70 ± 0.03	-
			Liver	1341.4 ± 66.7	5.22 ± 0.22	3.08 ± 0.16
			Spleen	162.2 ± 17.2	0.63 ± 0.05	0.37 ± 0.04
			Lungs	187.0 ± 12.3	0.73 ± 0.04	0.43 ± 0.02
			Thymus	84.2 ± 9.3	0.33 ± 0.03	0.19 ± 0.02
			Kidneys	362.6 ± 19.0	1.41 ± 0.05	0.83 ± 0.03
0.6 µg/ml	5	23.9 ± .55	Brain	431.2 ± 16.0	1.81 ± 0.10	-
			Liver	1254.4 ± 90.5	5.23 ± 0.27	2.95 ± 0.32
			Spleen	118.4 ± 17.7*	0.49 ± 0.07	0.28 ± 0.04
			Lungs	161.6 ± 15.4	0.67 ± 0.06	0.38 ± 0.04
			Thymus	90.8 ± 9.3	0.38 ± 0.04	0.21 ± 0.03
			Kidneys	363.4 ± 10.0	1.52 ± 0.02	0.85 ± 0.05
1.25 µg/ml	5	22.9 ± .97*	Brain	443.8 ± 12.7	1.94 ± 0.07	-
			Liver	1173.2 ± 42.6	5.13 ± 0.14	2.64 ± 0.06
			Spleen	119.2 ± 10.4*	0.53 ± 0.05*	0.27 ± 0.02
			Lungs	190.8 ± 12.8	0.83 ± 0.05	0.43 ± 0.02
			Thymus	62.8 ± 4.2	0.28 ± 0.02	0.14 ± 0.01
			Kidneys	341.6 ± 14.0	1.49 ± 0.05	0.77 ± 0.03

Values represent the mean ± SE derived from 5 animals per group. Those values whic differ significantly from the vehicle (tap water) at $p < .05$ are noted by an asterisk.

TABLE 78

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Hematological Parameters in Weanling Female Mice

Treatment	Hematocrit (%)	Hemoglobin (g%)	Erythrocytes (x 10^6/mm^3)	Leukocytes (x 10^3/mm^3)	Platelets (x 10^5/mm^3)
Vehicle	40.6 ± 0.5 (5)	12.6 ± 0.2 (5)	12.20 ± 0.68 (4)	9.59 ± 1.60 (4)	1.55 ± 0.13 (5)
0.1 µg/ml	41.0 ± 0.6 (3)	15.0 ± 2.0 (3)	13.43 ± 1.2 (3)	11.63 ± 1.60 (3)	1.66 ± 0.11 (3)
0.3 µg/ml	38.0 ± 1.4 (5)	11.0 ± 1.0 (5)	12.67 ± 1.2 (5)	7.50 ± 1.34 (5)	1.48 ± 0.22 (5)
0.6 µg/ml	41.4 ± 0.9 (5)	13.6 ± 0.3 (5)	13.68 ± 0.76 (5)	8.10 ± 0.49 (5)	1.92 ± 0.13 (5)
1.25 µg/ml	41.8 ± 0.6 (5)	11.7 ± 1.0 (5)	13.56 ± 1.1 (5)	6.16 ± 0.77 (5)	2.10 ± 0.29 (5)

Values represent the mean ± SE derived from the number of animals per group indicated in parentheses. The vehicle was tap water.

TABLE 79

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Hepatic Microsomal Hemoprotein Content in Weanling Female CD-1 Mice[a]

Treatment	Microsomal Protein (mg/g liver)	Cytochrome P450 (nmol/mg)[b]	Cytochrome b_5 (nmol/mg)[c]	Cytochrome P450 (nmol/g)[d]	Cytochrome b_5 (nmol/g)[e]
Vehicle	20.6 ± 0.8	0.629 ± 0.071	0.516 ± 0.038	12.9 ± 1.1	10.6 ± 0.5
0.1 µg/ml	26.2 ± 2.0	0.598 ± 0.040	0.483 ± 0.015	15.6 ± 1.5	12.6 ± 0.7
0.3 µg/ml	26.6 ± 1.2	0.755 ± 0.029	0.464 ± 0.037	20.1 ± 1.3	12.3 ± 0.8
0.6 µg/ml	27.9 ± 1.9*	0.741 ± 0.071	0.412 ± 0.038	20.6 ± 2.2	11.5 ± 1.4
1.25 µg/ml	30.7 ± 1.2*	0.662 ± 0.090	0.417 ± 0.032	20.7 ± 3.6	12.9 ± 1.4

[a] Values represent the mean ± SE derived from 5 animals per group. Those values which differ significantly from the vehicle control (tap water) at $p < .05$ are noted by an asterisk.

[b] nmol cytochrome P450/mg microsomal protein

[c] nmol cytochrome b_5/mg microsomal protein

[d] nmol cytochrome P450/g liver wet weight

[e] nmol cytochrome b_5/g liver wet weight

TABLE 80

Effects of Fourteen Day Exposure to Dexamethasone in Drinking Water on Hepatic Microsomal Enzyme Activity and Glutathione Content in Weanling Female CD-1 Mice[a]

Treatment	Aniline Hydroxylase (nmol/mg/min)[b]	Aminopyrine N-Demethylase (nmol/mg/min)[c]	Aniline Hydroxylase (nmol/g/min)[d]	Aminopyrine N-Demethylase (nmol/g/min)[e]	Glutathione (∂mol/g)[f]
Vehicle[g]	1.60 ± 0.16	11.30 ± 1.17	32.9 ± 2.1	231 ± 16	5.35 ± 0.31
0.1 µg/ml	1.57 ± 0.11	9.98 ± 0.66	41.2 ± 4.0	259 ± 15	5.63 ± 0.25
0.3 µg/ml	1.50 ± 0.14	9.02 ± 0.69	39.8 ± 3.5	239 ± 19	6.09 ± 0.32
0.6 µg/ml	1.64 ± 0.03	8.74 ± 0.48	45.0 ± 3.1	242 ± 12	4.52 ± 0.12
1.25 µg/ml	1.56 ± 0.09	8.69 ± 1.06	48.0 ± 3.8	269 ± 39	5.28 ± 0.22

[a]Values represent the mean ± SE derived from 5 animals per group.

[b]nmol p-aminophenol formed/mg microsomal protein

[c]nmol formaldehyde formed/mg microsomal protein

[d]nmol p-aminophenol formed/g liver wet weight

[e]nmol formaldehyde formed/g liver wet weight

[f]µmol reduced glutathione/g liver wet weight

[g]The vehicle was tap water.

TABLE 81

Time-Weighted Averages of Fluid and Chemical Consumption of Dexamethasone by CD-1 Mice in a Subchronic Ninety-Day Study[a]

Concentration (µg/ml)	DAYS 0-30		DAYS 31-60		DAYS 61-90		DAYS 0-90		
	ml/kg/day[b]	µg/kg/day[c]	ml/kg/day	µg/kg/day	ml/kg/day	µg/kg/day	ml/kg/day	µg/kg/day	ml/mouse/day[d]
				MALES					
0	220 ± 5	0	189 ± 2	0	177 + 2	0	197 ± 2	0	6.7 ± 0.1
0.2	219 ± 5	.04 ± .001	185 ± 4	.04 ± .001	187 ± 3	.04 ± .001	198 ± 4	.04 ± .001	6.6 ± 0.1
1.00	221 ± 8	.22 ± .01	205 ± 4	.20 ± .01	209 ± 6	.21 ± .01	212 ± 4	.21 ± .01	6.0 ± 0.2
2.00	229 ± 11	.46 ± .02	238 ± 8	.48 ± .02	213 ± 10	.43 ± .02	194 ± 16	.39 ± .03	5.1 ± 0.4
				FEMALES					
0	212 ± 6	0	199 ± 4	0	226 ± 8	0	212 ± 6	0	5.3 ± 0.1
0.2	190 ± 6	.04 ± .001	203 ± 4	.04 ± .001	199 ± 4	.04 ± .001	198 ± 4	.04 ± .001	4.7 ± 0.1
1.00	232 ± 9	.23 ± .01	214 ± 6	.21 ± .01	211 ± 4	.21 ± .01	216 ± 10	.22 ± .01	5.0 ± 0.3
2.00	206 ± 6	.41 ± .01	259 ± 7	.52 ± .01	240 ± 6	.48 ± .01	222 ± 11	.44 ± .02	4.7 ± 0.2

[a]Values represent the mean ± SE derived from 48 mice in the control group (deionized water) and 32 mice in the other groups.

[b]Average ml fluid consumed per kg body weight per day.

[c]Average µg of dexamethasone per kg body weight per day.

[d]Average ml fluid consumed per mouse per day.

TABLE 82

Body Weight Changes of CD-1 Mice Receiving Dexamethasone Phosphate in the Drinking Water for 90 Days

Concentration	Initial Weight+ (g)	Final Weight++ (g)	Delta Weight+++ (g)
		MALES	
0	26.1 ± 0.3	38.4 ± 0.5	12.2 ± 0.5
0.2 µg/ml	26.9 ± 0.4	38.8 ± 0.6	11.8 ± 0.5
1.0 µg/ml	25.2 ± 0.3	36.4 ± 0.8	11.2 ± 0.6
2.0 µg/ml	25.4 ± 0.4	34.8 ± 0.9	7.9 ± 1.1*
		FEMALES	
0	19.3 ± 0.2	28.0 ± 0.3	8.4 ± 0.4
0.2 µg/ml	17.9 ± 0.4	27.0 ± 0.6	9.1 ± 0.6
1.0 µg/ml	19.2 ± 0.4	25.2 ± 0.4	1.2 ± 0.7*
2.0 µg/ml	18.7 ± 0.3	23.1 ± 0.3	3.8 ± 0.6*

Values represent the mean ± SE derived from 48 mice in the control (deionized water) group and 32 mice in the other groups. Values which differ significantly from the control group at $p < 0.05$ are noted with an asterisk.

+Body weight on day exposure started.

++Body weight on last day of exposure.

+++Change in body weight over the 90 day exposure period.

The results of the gross pathology were largely unremarkable. The predominant feature was the reduced size of the spleen, and to a lesser extent, the thymus. Tissues were fixed in 10% formalin, but have not been prepared for histopathological analysis.

Body and organ weights of the mice are shown in Tables 83 and 84. Both sexes had decreased body weight at the two highest dose levels (1.0 and 2.0 µg/ml) up to almost 20% below vehicle controls. Liver weight was decreased in both sexes at all three dose levels when expressed in milligrams; however, when expressed as percent of body weight, no significant changes from control were seen. Spleen weights in both sexes were dramatically decreased after exposure to 1.0 or 2.0 µg/ml. This drop in spleen weight was seen when expressed in milligrams or as percent of body weight, and appears to be dose related. At the highest dose level of 2.0 µg/ml, spleen size was almost half the size of vehicle controls. Thymus weights were also decreased in a similar fashion in both sexes exposed to 1.0 or 2.0 µg/ml. Kidney weights increased in both sexes when expressed on a percent of body weight basis.

Hematological changes were surprisingly minimal (Tables 85 and 86). The hemoglobin values were below those for the historical controls and the male mice exposed to 1 µg/ml dexamethasone showed a 22% decrease. This is most likely not drug related. There was a dose-dependent reduction in leukocytes in male mice. The leukopenia was accompanied by a decrease in the percent of lymphocytes and an increase in the percent of polymorphonuclear leukocytes (Table 87). There was an 18% decrease in platelets of female mice exposed to the highest concentration of dexamethasone. The only coagulation factor that was altered by dexamethasone was fibrinogen; the males showed a 12% elevation in fibrinogen at the highest dose of dexamethasone.

The data summarizing the serum chemistry values are shown in Tables 88 and 89. The serum ions sodium, potassium, chloride, phosphorus and calcium showed no exposure-related effects. Serum protein was increased in female mice at the highest dose only. Blood glucose was decreased by 20% in both sexes at the highest dose of dexamethasone. There were no biologically relevant exposure-related effects on blood urea nitrogen. The serum enzymes were slightly elevated, particularly LDH and SAP. SGOT was elevated 39% in males, and SGPT was elevated 58% in females.

The liver appears to be one target for dexamethasone when administered in the drinking water. With respect to hepatic microsomal activities, the females

TABLE 83

Body and Organ Weights of Male CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	DEXAMETHASONE 1.0 µg/ml	DEXAMETHASONE 2.0 µg/ml
Body Weight (g)	40.7 ± 0.5	40.9 ± 0.9	34.4 ± 1.0*	32.2 ± 0.9*
Brain (mg)	496 ± 6	483 ± 5	500 ± 10	502 ± 8
(% body weight)	(1.22)	(1.19)	(1.46)*	(1.57)*
Liver (mg)	2259 ± 57	1987 ± 54*	1782 ± 91*	1680 ± 62*
(% body weight)	(5.55)	(4.86)*	(5.19)	(5.21)
Spleen (mg)	191 ± 14	161 ± 10	116 ± 8*	88 ± 6*
(% body weight)	(0.47)	(0.40)*	(0.33)*	(0.27)*
Lungs (mg)	236 ± 7	226 ± 5	214 ± 7*	215 ± 6*
(% body weight)	(0.58)	(0.56)	(0.62)	(0.67)*
Thymus (mg)	52 ± 4	52 ± 3	36 ± 3*	33 ± 2*
(% body weight)	(0.13)	(0.13)	(0.10)	(0.10)
Kidneys (mg)	699 ± 20	656 ± 25	682 ± 25	654 ± 16
(% body weight)	(1.72)	(1.60)	(1.99)*	(2.04)*
Testes (mg)	270 ± 9	272 ± 12	252 ± 12	276 ± 16
(% body weight)	(0.66)	(0.67)	(0.73)	(0.86)*

The numbers represent the mean ± SE of 20 mice in the vehicle group, 16 in the 0.2 µg/ml group, 12 in the 1.0 µg/ml group, and 9 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

TABLE 84

Body and Organ Weights of Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	DEXAMETHASONE 1.0 µg/ml	DEXAMETHASONE 2.0 µg/ml
Body Weight (g)	28.6 ± 0.4	28.3 ± 1.0	26.4 ± 0.5*	24.0 ± 0.8*
Brain (mg)	522 ± 6	504 ± 7	521 ± 6	541 ± 8*
(% body weight)	(1.84)	(1.80)	(1.98)*	(2.28)*
Liver (mg)	1399 ± 48	1233 ± 53*	1180 ± 36*	1240 ± 42*
(% body weight)	(4.92)	(4.36)	(4.45)	(5.24)
Spleen (mg)	126 ± 7	125 ± 8	91 ± 6*	71 ± 5*
(% body weight)	(0.44)	(0.44)	(0.34)*	(0.30)*
Lungs (mg)	206 ± 4	192 ± 6	195 ± 5	202 ± 7
(% body weight)	(0.72)	(0.68)	(0.74)	(0.85)*
Thymus (mg)	48 ± 2	46 ± 2	37 ± 2*	32 ± 2*
(% body weight)	(0.17)	(0.16)	(0.14)*	(0.13)*
Kidneys (mg)	392 ± 9	372 ± 15	401 ± 12	397 ± 10
(% body weight)	(1.38)	(1.32)	(1.51)*	(1.67)*

The numbers represent the mean ± SE of 23 mice in the vehicle group, 16 in the 0.2 µg/ml group, 16 in the 1.0 µg/ml group, and 15 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

TABLE 85

Hematology and Blood Coagulation Parameters of Male CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	1.0 µg/ml	2.0 µg/ml
Hematocrit (%)	42.0 ± 0.7	42.8 ± 0.7	43.0 ± 0.6	43.8 ± 0.7
Hemoglobin (g%)	11.1 ± 0.3	10.7 ± 0.3	8.7 ± 0.3*	10.9 ± 0.4
Erythrocytes ($10^6/mm^3$)	8.09 ± 0.19	7.88 ± 0.19	7.99 ± 0.16	8.20 ± 0.24
Leukocytes ($10^3/mm^3$)	7.00 ± 0.49	5.54 ± 0.35*	5.26 ± 0.55*	5.11 ± 0.49*
Platelets ($10^5/mm^3$)	3.88 ± 0.12	3.86 ± 0.34	3.48 ± 0.11	3.46 ± 0.17
Fibrinogen (mg%)	304 ± 10	286 ± 7	308 ± 12	342 ± 12*
Prothrombin Time (seconds)	9.8 ± 0.1	9.8 ± 0.2	10.3 ± 0.3	9.7 ± 0.2
APTT (seconds)	30.7 ± 0.9	30.4 ± 1.2	28.9 ± 1.5	31.8 ± 2.0

The numbers represent the mean ± SE of 20 mice in the vehicle group, 14 in the 0.2 µg/ml group, 12 in the 1.0 µg/ml group, and 9 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

TABLE 86

Hematology and Blood Coagulation Parameters
of Female CD-1 Mice Exposed to Dexamethasone
in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	1.0 µg/ml	2.0 µg/ml
Hematocrit (%)	40.9 ± 0.5	42.4 ± 0.6	41.7 ± 0.4	41.6 ± 0.6
Hemoglobin (g%)	10.4 ± 0.3	9.6 ± 0.4	9.2 ± 0.4	10.0 ± 0.4
Erythrocytes ($10^6/mm^3$)	7.41 ± 0.14	7.77 ± 0.14	7.56 ± 0.16	7.24 ± 0.13
Leukocytes ($10^3/mm^3$)	4.73 ± 0.31	4.74 ± 0.28	4.09 ± 0.28	3.73 ± 0.29*
Platelets ($10^5/mm^3$)	4.04 ± 0.13	3.85 ± 0.19	3.88 ± 0.15	3.30 ± 0.19*
Fibrinogen (mg%)	212 ± 5	219 ± 7	212 ± 5	212 ± 6
Prothrombin Time (seconds)	9.8 ± 0.1	9.9 ± 0.2	9.8 ± 0.2	9.8 ± 0.1
APTT (seconds)	35.2 ± 1.2	33.8 ± 1.7	32.2 ± 1.2	32.0 ± 1.4

The numbers represent the mean ± SE of 23 mice in the vehicle group, 16 in the 0.2 µg/ml group, 16 in the 1.0 µg/ml group, and 15 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

TABLE 87

Differential Leukocyte Analysis of CD-1 Mice Expsoed to Dexamethasone in the Drinking Water for 90 Days

Concentration	Lymphocytes (%)	Polymorphonuclear Leukocytes (%)	Monocytes (%)	Eosinophils (%)
		MALES		
Distilled Water	72 ± 3	24 ± 3	4 ± 1	1 ± 0.5
0.2 µg/ml	70 ± 3	22 ± 3	4 ± 1	1 ± 0.3
1.0 µg/ml	57 ± 3*	37 ± 3*	5 ± 1	1 ± 0.4
2.0 µg/ml	37 ± 3*	58 ± 3*	4 ± 1	1 ± 0.5
		FEMALES		
Distilled water	79 ± 2	16 ± 2	4 ± 1	1 ± 0.3
0.2 µg/ml	83 ± 2	12 ± 1	3 ± 1	2 ± 0.5
1.0 µg/ml	75 ± 2	21 ± 2	2 ± 1	2 ± 1.0
2.0 µg/ml	56 ± 4*	40 ± 4*	3 ± 1	2 ± 0.5

Values represent the mean ± SE derived from 20 mice in the distilled water control group and 15 mice in each of the dexamethasone-exposed groups. The values which differ significantly from control at $p < 0.05$ are noted by an asterisk.

TABLE 88

Serum Chemistry Values of Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months[a]

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	1.0 µg/ml	2.0 µg/ml
Calcium (mg%)	14.0 ± 0.3	13.9 ± 0.5	13.6 ± 0.6	13.4 ± 0.5
Sodium (mEq/L)	148 ± 1	154 ± 2*	154 ± 1*	152 ± 4
Chloride (mEq/L)	104 ± 1	100 ± 1	99 ± 2*	97 ± 8
Potassium (mEq/L)	6.60 ± 0.16	6.84 ± 0.21	6.89 ± 0.16	7.09 ± 0.35
Protein (g%)	8.78 ± 0.17	9.13 ± 0.17	9.17 ± 0.19	9.66 ± 0.32*
Glucose (mg%)	144 ± 3	154 ± 4	139 ± 5	118 ± 8*
Cholesterol (mg%)	175 ± 23	204 ± 33	191 ± 29	290 ± 21
Phosphorus (mg%)	10.8 ± 0.3	9.9 ± 0.3	10.1 ± 0.3	8.7 ± 0.4*
BUN[b] (mg%)	22.7 ± 1.0	23.1 ± 1.7	23.1 ± 2.0	23.0 ± 1.8
LDH[c] (IU/L)	694 ± 47	762 ± 98	697 ± 41	1076 ± 144*
SGPT[d] (IU/L)	29.5 ± 1.2	30.7 ± 1.2	32.4 ± 1.7	46.5 ± 6.0*
SGOT[e] (IU/L)	95.9 ± 5.1	98.9 ± 6.4	112.3 ± 4.6	116.6 ± 8.8
SAP[f] (IU/L)	81.4 ± 4.6	77.1 ± 4.8	103.2 ± 8.0*	90.0 ± 8.4

[a]The numbers represent the mean ± SE of 24 mice in the vehicle group, 16 in the 0.2 µg/ml group, 16 in the 1.0 µg/ml group, and 12 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

[b]BUN = blood urea nitrogen

[c]LDH = lactic dehydrogenase

[d]SGPT = serum glutamic pyruvic transaminase

[e]SGOT = serum glutamic oxaloacetic transaminase

[f]SAP = serum alkaline phosphatase

TABLE 89

Serum Chemistry Values of Male CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months[a]

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	DEXAMETHASONE 1.0 µg/ml	DEXAMETHASONE 2.0 µg/ml
Calcium (mg%)	15.3 ± 0.4	15.2 ± 0.5	14.9 ± 0.6	14.6 ± 0.9
Sodium (mEq/L)	153 ± 1	150 ± 2	153 ± 2	156 ± 2
Chloride (mEq/L)	100 ± 1	102 ± 1	100 ± 2	105 ± 3
Potassium (mEq/L)	7.63 ± 0.20	7.94 ± 0.18	8.19 ± 0.23	8.20 ± 0.31
Protein (g%)	8.36 ± 0.17	8.81 ± 0.23	8.29 ± 0.21	8.12 ± 0.14
Glucose (mg%)	145 ± 4	151 ± 4	134 ± 4	112 ± 9*
Cholesterol (mg%)	227 ± 25	246 ± 37	243 ± 34	290 ± 64
Phosphorus (mg%)	10.9 ± 0.1	11.2 ± 0.2	10.2 ± 0.2*	10.3 ± 0.4
BUN[b] (mg%)	22.4 ± 0.7	19.3 ± 1.0*	18.9 ± 0.7*	20.0 ± 1.4
LDH[c] (IU/L)	737 ± 68	571 ± 60	719 ± 83	1049 ± 150*
SGPT[d] (IU/L)	32.5 ± 1.9	30.8 ± 2.9	28.3 ± 1.2	36.1 ± 2.1
SGOT[e] (IU/L)	75.9 ± 3.1	75.3 ± 3.0	78.1 ± 4.1	106.3 ± 9.6*
SAP[f] (IU/L)	32.6 ± 3.2	36.1 ± 2.6	56.8 ± 7.0*	81.0 ± 19.1*

[a]The numbers represent the mean ± SE of 24 mice in the vehicle group, 15 in the 0.2 µg/ml group, 16 in the 1.0 µg/ml group, and 9 in the 2.0 µg/ml group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

[b]BUN = blood urea nitrogen

[c]LDH = lactic dehydrogenase

[d]SGPT = serum glutamic pyruvic transaminase

[e]SGOT = serum glutamic oxaloacetic transaminase

[f]SAP = serum alkaline phosphatase

were the most sensitive (Tables 90 and 91). Microsomal protein, based on mg/g liver, was increased, as was cytochrome P450. There was a significant decrease in aniline hydroxylase in females at the middle dose. In the males, there was an induction of aminopyrine demethylase.

The change most suggestive of liver damage was the dose-dependent reduction in liver glutathione (non-protein sulhydryl containing compound). This was manifested in a 33% and 24% decrease in males and females receiving the highest concentration of dexamethasone, respectively.

Based on the more routine toxicological parameters, the most sensitive indicators of toxcicity were changes in body, spleen and thymus weights, and depression of peripheral leukocyte count.

TABLE 90

Hepatic Microsomal Activities in Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	DEXAMETHASONE 1.0 µg/ml	DEXAMETHASONE 2.0 µg/ml
Microsomal Protein (mg/g liver)	21.4 ± 1.0	24.0 ± 0.5	25.6 ± 1.0*	27.1 ± 0.8*
Cytochrome P-450 (nmol/mg protein)	.941 ± .038	.919 ± .033	.935 ± .018	1.100 ± .067*
Cytochrome b_5 (nmol/mg protein)	.561 ± .023	.523 ± .020	.577 ± .032	---
Glutathione (nmol/g liver)	5.80 ± .45	5.23 ± .18	4.68 ± .27*	3.91 ± .16*
Aminopyrine N-demethylase (nmol/mg/min)	15.10 ± .50	13.64 ± .46	14.99 ± .59	15.51 ± .80
Aniline Hydroxylase (nmol/mg/min)	2.30 ± .08	2.12 ± .08	1.95 ± .08*	2.07 ± .05

The numbers represent the mean ± SE of 8 mice per group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

TABLE 91

Hepatic Microsomal Activities in Male CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Parameter	Vehicle	DEXAMETHASONE 0.2 µg/ml	DEXAMETHASONE 1.0 µg/ml	DEXAMETHASONE 2.0 µg/ml
Microsomal Protein (mg/g liver)	22.9 ± 1.6	24.9 ± 0.6	23.6 ± 0.8	25.0 ± 0.6
Cytochrome P-450 (nmol/mg protein)	1.53 ± .11	1.59 ± .02	1.36 ± .07	1.50 ± .07
Cytochrome b_5 (nmol/mg protein)	.450 ± .016	.485 ± .015	.421 ± .025	.447 ± .016
Glutathione (nmol/g liver)	6.89 ± .24	6.07 ± .18*	5.90 ± .12*	5.25 ± .18*
Aminopyrine N-demethylase (nmol/mg/min)	9.20 ± .77	9.99 ± .31	12.36 ± .35*	12.84 ± .57*
Aniline Hydroxylase (nmol/mg/min)	2.22 ± .08	2.30 ± .08	2.08 ± .11	2.06 ± .07

The numbers represent the mean ± SE of 8 mice per group. Those values which differ significantly from the vehicle (deionized water) group at $p < .05$ are noted by an asterisk.

E. IMMUNOTOXICOLOGY OF DEXAMETHASONE

Introduction

The previous section was concerned with general toxicological effects of subchronic dexamethasone exposure in the CD-1 mouse. The most pronounced effects attributed to dexamethasone exposure were a decrease in body weight gain, decreases in spleen and thymus weight, a decrease in peripheral leukocyte count and a depletion of liver glutathione.

Presented against this background of data, this section reports the effects of subchronic dexamethasone exposure on the immune system of the mouse. The parameters assessed were humoral immunity, cell-mediated immunity, lymphocyte responsiveness to mitogens and the functions of bone marrow cells and the reticuloendothelial system.

Materials and Methods

Details of the methods of procedure for the immunological assays and historical controls have been fully described in the section entitled "General Approach and Methods". The exposure procedure and methodology for dexamethasone was described in the previous section.

Humoral Immune Response of Mice Exposed to Dexamethasone in the Drinking Water

The humoral immune response to sRBC was measured in male and female mice following exposure to three levels of dexamethasone in the drinking water (Tables 92 and 93). The number of IgM spleen antibody-forming cells (AFC) produced against sheep erythrocytes was measured on Day 4 (which was the peak day of response) and on Day 5 after immunization. The immunization was carried out on Days 85 and 86 of dexamethasone exposure. On peak day of response, the AFC per spleen response was strikingly decreased in both male and female mice exposed to 1.0 and 2.0 μg/ml dexamethasone. In males, the decrease was 34% and 55%, and in females the suppression was 59% and 67%. The response on Day 5 showed a similar pattern, except that a 32% suppression was also manifested at the lowest concentration in the female mice. Also, on this day the specific activity (i.e., AFC/10^6 spleen cells) of male mice was also reduced in a dose-dependent manner. Therefore, on the peak day of response, the reduction in AFC was a function of loss of spleen cells. This is corroborated by the decrease in spleen weight (Tables 83 and 84) and spleen cell number (Table 92).

TABLE 92

Spleen Antibody Forming Cell Response to Sheep Erythrocytes

in CD-1 Mice Exposed to Dexamethasone for Three Months

Concentration	DAY 4 cells/spleen x 10^7	DAY 4 10^5 AFC/Spleen	DAY 4 AFC/10^6	DAY 5 cells/spleen x 10^7	DAY 5 10^5 AFC/Spleen	DAY 5 AFC/10^6
			MALES			
Vehicle	18.9 ± 0.7	3.31 ± 0.28	1728 ± 96	17.0 ± 1.0	1.85 ± 0.15	1091 ± 69
0.2 µg/ml	19.7 ± 0.7	3.66 ± 0.40	1858 ± 174	16.4 ± 1.5	1.59 ± 0.18	973 ± 81
1.0 µg/ml	11.6 ± 0.8*	2.20 ± 0.28*	1870 ± 193	9.1 ± 0.8*	0.73 ± 0.12*	780 ± 109*
2.0 µg/ml	7.8 ± 0.8*	1.48 ± 0.38*	1797 ± 294	5.3 ± 0.1*	0.36 ± 0.05*	687 ± 100*
			FEMALES			
Vehicle	14.8 ± 0.6	4.56 ± 0.53	3039 ± 336	14.4 ± 1.0	2.28 ± 0.23	1617 ± 152
0.2 µg/ml	14.3 ± 0.8	3.98 ± 0.49	2731 ± 250	13.1 ± 0.9	1.54 ± 0.19*	1173 ± 124
1.0 µg/ml	8.4 ± 0.5*	1.89 ± 0.35*	2156 ± 357	9.0 ± 0.7	1.30 ± 0.23*	1445 ± 205
2.0 µg/ml	6.6 ± 0.6*	1.50 ± 1.91*	2364 ± 327	5.7 ± 0.5	0.87 ± 0.12*	1508 ± 95

The numbers represent the mean ± SE of 12 mice in the vehicle (deionized water) group, and 5-8 mice in the other groups. Those values which differ significantly from the vehicle at $p < .05$ are noted by an asterisk.

TABLE 93

Hemagglutination Titers in CD-1 Mice Exposed tc Dexamethasone in the Drinking Water for Three Months

Concentration	MALES No. Mice	MALES Log_2 Titer	FEMALES No. Mice	FEMALES Log_2 Titer
Vehicle	20	9.622 ± 0.105	21	9.512 ± 0.112
0.2 µg/ml	12	9.739 ± 0.149	15	9.722 ± 0.131
1.0 µg/ml	11	9.322 ± 0.135	16	9.135 ± 0.188
2.0 µg/ml	8	8.697 ± 0.183*	13	8.707 ± 0.213*

Hemagglutination titers are expressed as log_2 of the reciprocal of the dilution shown not to agglutinate. The numbers represent the mean ± SE of the number of animals shown. Values which differ significantly from the vehicle control (deionized water) group at $p < .05$ are noted with an asterisk.

Serum hemagglutinating antibody levels were also reduced. This assay measures both serum IgM and IgG. Decreased titers were seen in both sexes at the 2.0 μg/ml dexamethasone exposure level. The data are presented as LOG_2 of the reciprocal of the titer. When reconverted to titer, the suppression was 47% for the males and 43% for the females (Table 93).

Cell-Mediated Immune Response of Mice Exposed to Dexamethasone in the Drinking Water

Three assays used to evaluate the overall status of cellular immunity were the DTH response to sRBC, popliteal lymph node proliferation in response to footpad sensitization to sRBC, and spleen cell response to the T-lymphocyte mitogen, concanavalin A (Tables 94 and 95). There was a dose-dependent suppression in the DTH response in both males and females, with the mice exposed to the highest concentration (i.e., 2.0 μg/ml) showing a suppression of 64% and 74%. With respect to popliteal lymph node proliferation, no dose dependency was seen, but mice exposed to the highest concentration of dexamethasone showed a 23% and 28% suppression. These data indicate that dexamethasone-exposed mice have a marked reduction in their ability to elicit an immune-mediated inflammatory response, and to a lesser extent, a reduced ability to mount a response on the afferent arm of the immune system.

Lymphocyte Response to Mitogens

Although there was a decrease in the number of spleen cells and overall suppression of humoral and cell-mediated immunity, the cells remaining in the spleen responded to the B cell mitogen similarly to controls (Table 95). The concanavalin A response was increased 29%, 9% and 273% above control values for the three concentrations of the mitogen. The most magnified effect was observed at the highest concentration of Con A, which is on the descending portion of the mitogen dose-response curve.

Functional Activity of the Reticuloendothelial System

The functional activity of the fixed macrophages, particularly the liver, spleen and lungs was assessed by measuring the vascular clearance rate and tissue uptake of ^{51}Cr-labelled sRBC (Tables 96 and 97). There was a dose-dependent suppression in hepatic uptake of ^{51}Cr sRBC in female mice, measured as uptake per organ and per mg of organ. There was a dose-dependent increase

TABLE 94

Cell-Mediated Immune Response to Sheep Erythrocytes in CD-1 Mice

Exposed to Dexamethasone for Three Months

	STIMULATION INDEX			
	MALES		FEMALES	
Concentration	Footpad Swelling	Popliteal Lymph Nodes	Footpad Swelling	Popliteal Lymph Nodes
Distilled Water	6.1 ± 0.5 (15)	11.2 ± 0.9 (12)	6.0 ± 0.5 (15)	12.8 ± 1.1 (12)
0.2 µg/ml	5.4 ± 0.8 (10)	11.6 ± 1.1 (12)	5.2 ± 0.9 (11)	9.6 ± 1.2 (12)
1.0 µg/ml	4.7 ± 0.5 (12)	12.4 ± 0.9 (11)	4.1 ± 0.8 (11)	10.8 ± 0.9 (10)
2.0 µg/ml	2.2 ± 0.7* (10)	8.6 ± 1.3* (6)	1.6 ± 0.5* (11)	8.4 ± 0.8* (11)

Stimulation indices for footpad swelling and popliteal lymph node proliferation were calculated as described in Methods. The value for unsensitized footpad controls was 1.6 for males and 2.4 for females; the unsensitized popliteal lymph node value was 1.1 for males and 1.3 for females. The values represent the mean ± SE derived from the numbers in parenthesis. Those values which differ significantly from the distilled water control at $p < .05$ are noted with an asterisk.

TABLE 95

Lymphocyte Responsiveness to Concanavalin A and Bacterial Lipopolysaccharide Using Spleen Cells from Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Exposure Group	cpm/5 x 10^5 cells	
	Concanavalin A	Lipopolysaccharide
	1 µg/5 x 10^5 cells	1 µg/5 x 10^5 cells
Deionized Water	126485 ± 1992	104684 ± 6783
0.2 µg/ml	125666 ± 1999	77423 ± 4288
1.0 µg/ml	138826 ± 3730	85519 ± 6599
2.0 µg/ml	163377 ± 6465*	96819 ± 11859
	5 µg/5 x 10^5 cells	5 µg/5 x 10^5 cells
Deionized Water	270655 ± 4365	120114 ± 8021
0.2 µg/ml	290948 ± 7555	89250 ± 5961
1.0 µg/ml	284667 ± 5001	97818 ± 7263
2.0 µg/ml	297623 ± 6447*	102512 ± 11668
	10 µg/5 x 10^5 cells	20 µg/5 x 10^5 cells
Deionized Water	24762 ± 6488	100549 ± 8877
0.2 µg/ml	19661 ± 2509	75858 ± 7349
1.0 µg/ml	32576 ± 4488	94676 ± 9683
2.0 µg/ml	67786 ± 13795*	102779 ± 13612

Values represent the mean cpm ± SE derived from 12-15 mice per group. Those values which differ significantly from deionized water control at $p < .05$ are noted by an asterisk. CPM/5 x 10^5 cells in the absence of mitogen ranged from 3930 - 5650.

TABLE 96

Functional Activity of the Reticuloendothelial System in Female CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Exposure Group	Body Weight (g)	Phagocytic Index	Organ	Weight (mg)	% Uptake	cpm/mg
Vehicle	31.9 ± 0.9	.10 ± .01	Liver	1494 ± 50	62.0 ± 2.7	333 ± 14
			Spleen	151 ± 5	9.8 ± 2.3	527 ± 123
			Lungs	255 ± 18	1.1 ± 0.3	32 ± 6
			Thymus	54 ± 4	.007 ± .001	5 ± 1
			Kidneys	418 ± 16	1.8 ± 0.2	35 ± 3
0.2 µg/ml	30.6 ± 1.2	.11 ± .01	Liver	1377 ± 99	62.5 ± 2.0	353 ± 16
			Spleen	140 ± 13	9.7 ± 1.7	579 ± 128
			Lungs	307 ± 36	0.7 ± 0.1	18 ± 3
			Thymus	66 ± 8	.008 ± .001	4 ± 1
			Kidneys	426 ± 22	1.9 ± 0.3	34 ± 5
1.0 µg/ml	26.7 ± 0.9*	.10 ± .01	Liver	1268 ± 96*	49.7 ± 4.8*	262 ± 20*
			Spleen	167 ± 22	13.5 ± 1.2	623 ± 115
			Lungs	338 ± 25*	1.1 ± 0.2	21 ± 3
			Thymus	-	-	-
			Kidneys	447 ± 28	2.4 ± 0.5	39 ± 11
2.0 µg/ml	23.7 ± 0.7*	.08 ± .01	Liver	990 ± 39*	43.3 ± 3.4*	258 ± 18*
			Spleen	65 ± 7*	10.3 ± 1.1	930 ± 66*
			Lungs	223 ± 11	1.5 ± 0.1	41 ± 4
			Thymus	34 ± 4	.006 ± .001	-
			Kidneys	362 ± 11*	3.3 ± 0.4*	54 ± 8

Values represent the mean ± SE derived from 7-11 mice per group. Values which differ significantly from the vehicle (deionized water) control group at $p < .05$ are noted with an asterisk.

TABLE 97

Functional Activity of the Reticuloendothelial System in Male CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Exposure Group	Body Weight (g)	Phagocytic Index	Organ	Weight (mg)	% Uptake	cpm/mg
Vehicle	36.6 ± 1.0	.12 ± .01	Liver	1798 ± 80	60.6 ± 1.5	313 ± 13
			Spleen	134 ± 9	8.6 ± 1.0	609 ± 72
			Lungs	336 ± 27	1.2 ± 0.3	37 ± 10
			Thymus	53 ± 5	.006 ± .001	6 ± 1
			Kidneys	558 ± 15	2.2 ± 0.3	35 ± 4
0.2 µg/ml	36.0 ± 0.3	.11 ± .01	Liver	1827 ± 37	57.4 ± 3.3	285 ± 20
			Spleen	157 ± 19	11.2 ± 1.0	689 ± 84
			Lungs	379 ± 29	1.8 ± 1.2	48 ± 32
			Thymus	66 ± 20	.007 ± .002	5 ± 1
			Kidneys	604 ± 32	2.0 ± 0.5	33 ± 11
1.0 µg/ml	29.9 ± 0.7*	.10 ± .01	Liver	1436 ± 26*	57.4 ± 2.2	299 ± 12
			Spleen	102 ± 10	6.8 ± 1.5	501 ± 100
			Lungs	271 ± 15	0.8 ± 0.1	22 ± 1
			Thymus	60 ± 10	.008 ± .001	5 ± 2
			Kidneys	595 ± 18	1.6 ± 0.3	21 ± 3
2.0 µg/ml	28.5 ± 0.6	.08 ± .01	Liver	1288 ± 35*	48.2 ± 4.5*	265 ± 21
			Spleen	100 ± 20	10.4 ± 1.6	938 ± 188
			Lungs	273 ± 14	1.6 ± 0.2	43 ± 6
			Thymus	42 ± 7	.006 ± .001	13 ± 5
			Kidneys	545 ± 17	3.0 ± 0.5	38 ± 6

Values represent the mean ± SE derived from 7-12 mice per group. Values which differ significantly from the vehicle (deionized water) control group at $p < .05$ are noted with an asterisk.

in uptake of sRBC in the spleen in females only when expressed as cpm/mg. Although the same trends occurred in males, the only significant change was a suppression of hepatic phagocytosis.

Bone Marrow

The foundation of the immune system is the bone marrow, which gives rise to the immunocompetent cells. Three indicators of toxicity to the bone marrow were used. They included cellularity, DNA synthesis and bone marrow stem cells (CFU-GM). There was a slight reduction in the number of cells per femur, which was significant in the female mice, and was related to an increase in the number of stem cells (granulocyte/monocyte) (Table 98). These data indicate that the cells lost from the bone marrow were CFU-GM stem cells. There was no dose-dependent or biological effect on bone marrow DNA synthesis (Table 99).

Based on the immunological assays, mice exposed to dexamethasone were deficient in humoral and cell-mediated immunity, with minimal effects on the macrophage and bone marrow parameters.

TABLE 98

Bone Marrow Cellularity and Stem Cells in CD-1 Mice Exposed to Dexamethasone in the Drinking Water for 90 Days

Exposure Group	MALES			FEMALES		
	Number Mice	Cells/femur x 10^7	CFU-GM 10^5 cells	Number Mice	Cells/femur x 10^5	CFU-GM 10^5 cells
Dist. Water	20	1.8 ± 0.1	203 ± 1	23	1.1 ± 0.1	232 ± 1
0.2 µg/ml	11	2.0 ± 0.1	207 ± 2	16	1.2 ± 0.1	234 ± 2
1.0 µg/ml	10	1.7 ± 0.1	182 ± 2*	16	1.0 ± 0.1	228 ± 2
2.0 µg/ml	7	1.5 ± 0.1	198 ± 3	15	0.9 ± 0.1*	254 ± 2

The formation of colonies in soft agar was used to enumerate the bone marrow CFU-GM stem cells. Values are expressed as mean ± SE and those which differ significantly from the control (distilled water) group at $p < 0.05$ are noted with an asterisk.

TABLE 99

Bone Marrow DNA Synthesis in CD-1 Mice Exposed to Dexamethasone in the Drinking Water for Three Months

Exposure Group	MALES			FEMALES		
	60 Min.	120 Min.	180 Min.	60 Min.	120 Min.	180 Min.
Distilled Water	2900 ± 153	3996 ± 260	4290 ± 254	4129 ± 270	5081 ± 332	5709 ± 329
0.2 µg/ml	3792 ± 208*	5208 ± 396*	5927 ± 366*	5233 ± 297*	6280 ± 292*	6701 ± 383
1.0 µg/ml	3666 ± 391*	4808 ± 455	5175 ± 482	5624 ± 292*	5531 ± 210	6097 ± 262*
2.0 µg/ml	3820 ± 320*	5128 ± 376	5277 ± 338	4728 ± 210	5847 ± 308	6414 ± 295

DNA synthesis was estimated from incorporation of ^{125}I-Iododeoxyuridine, as described in Methods. The values in this table represent the mean ± SE from six replicate samples of each animal. There were 20 mice in the distilled water group and 7-15 mice in the other groups. Uptake was determined after 60, 120, and 180 minutes. Values which differ from the distilled water control at $p < .05$ are noted by an asterisk.

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